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Cardiotoxins (CTXs) and melittin are potent membrane perturbing agents that interact in mutual synergism with phospholipase A2 (PLA_2). The present studies support the hypothesis that there is a hemolytic component of at least one of the CTXs *not* dependent on PLA_2 activity, but that is enhanced by PLA_2 contamination. Melittin and CTX elevate levels of diglycerides in airway epithelial cells (melittin) and skeletal muscle (melittin, CTX). The effects of CTX on normal muscle cannot be attributed to activation of tissue phospholipase A_2 activity, as suggested by other investigators. The present study demonstrates that melittin does not at all enhance the action of bee venom PLA_2 on mixed micelle substrates in the absence of NaCl, but does increase bee venom PLA_2 activity on mixed micelles in the presence of NaCl. Melittin and CTX act by similar, but not identical mechanisms, based on similarities and differences in their interactions with PLA_2 and differences in effects on lipid metabolism in cell cultures. Certainly bee venom and snake venom PLA_2s differ markedly in their interactions with CTXs and melittin.

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MODE OF ACTION OF MEMBRANE PERTURBING AGENTS: SNAKE VENOM CARDIOTOXINS AND PHOSPHOLIPASES A

ANNUAL REPORT

JEFFREY E. FLETCHER

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Problem Under Study:

Contract No. DAMD17-87-C-7155 addresses the mode of action of snake venom cardiotoxins (CTXs) and the interaction of these toxins with a second snake venom component, phospholipase A_2 (PLA_2). Studies on presynaptically-acting snake venom PLA_2 neurotoxins examine whether similarities exist in mechanisms between these toxins and the CTXs, or if the CTXs act synergistically with the PLA_2 neurotoxins. CTXs are potent membrane perturbing agents and PLA_2s hydrolyze diacylphosphoglycerides at the two position, generating two biologically active metabolites - free fatty acids and lysophospholipids. The CTXs and PLA_2s act in synergy to induce hemolysis of red blood cells and skeletal muscle contractures. The CTXs and PLA_2s are of special interest due to the mutual potentiation observed; that is, the hemolytic activity of CTX is greatly increased by PLA_2 and the hydrolytic activity of PLA_2 is greatly increased by CTX. Combinations of these agents with other toxins, such as the presynaptically-acting snake venom neurotoxins, which all possess PLA_2 activity, would result in novel potent biological warfare approaches. Additionally, based on our recent studies, there are similarities between the modes of action of CTX and presynaptically- acting snake venom toxins. Therefore, a greater understanding of the modes of action of venom CTXs and PLA_2s and their interactions has important military significance.

The specific problems addressed in this contract are:

1. What are the effects of CTX alone and CTX-PLA_2 combinations on contracture induction of human and rat skeletal muscle and on Ca^{2+} transients in human and equine lymphocytes?
2. What is the dependence of CTX action on fatty acid distribution and the free fatty acid content of muscle and red blood cells?
3. What are the hemolytic effects of CTX alone and CTX-PLA_2 combinations in red blood cells from different species?
4. What are the effects of CTX on endogenous lipolytic enzymes in skeletal muscle and red blood cells?
5. What is the role of toxin internalization in the action of CTX?
6. What are the similarities and differences between CTXs and the presynaptically-acting snake venom PLA_2s?

Background:

Early studies in the isolation of protein components from snake venoms identified a number of toxic low molecular weight polypeptides (ca. 6000 MW). These toxins were identified in a number of laboratories and, because they affected a large number of systems, some confusion arose as to the true site of action of the toxins. The polypeptides were named according to their observed toxic actions and included: cardiotoxins, which arrested the heart in systole (Sarkar, 1951; Lee et al., 1968); Cobramine A and B (Larsen and Wolff, 1968); cytotoxins (Braganca et al., 1967) and; direct-lytic factors (Condrea et al., 1964). It later became apparent that these seemingly different toxins indeed shared similar mechanisms when compared on the same assay systems. The multitude of different effects possessed by each of these toxins were suggested to be best described by the more general term membrane-active polypeptides (Condrea, 1974). However, the toxins have since been

grouped together under the original name, cardiotoxin (CTX). CTXs are now regarded as low molecular weight basic polypeptides that, among other effects, depolarize and induce contractures in muscle (Chang, 1979) and hemolyze red blood cells (Condrea, 1974; 1979). There appears to be considerable amino acid sequence homology among the CTXs (Condrea, 1974). Despite considerable research in this area, the specific mechanism(s) of action of the CTXs is(are) unknown (Chang, 1979; Harvey; 1985).

Phospholipase A_2 (PLA_2; EC3.1.1.4; ca. 12,000 MW) is a major component of bee and snake venom. PLA2 is a Ca^{2+}-dependent enzyme (Shipolini et al., 1971; Tsai et al., 1985) that catalyzes the hydrolysis of phospholipids at the #2 position releasing lysophospholipids and, primarily, unsaturated free fatty acids (Hanahan et al., 1960). The bee and cobra (*Naja naja*) venom PLA_2 enzymes readily hydrolyze biological phospholipid substrates, but are unable to penetrate membrane bilayers (Zwaal et al., 1975; Sundler et al., 1978; Fletcher et al., 1987). The inability to hydrolyze the inner phospholipids of the bilayer does not relate to interactions of these primarily negatively-charged phospholipids with spectrin (Raval and Allan, 1984), and can even be observed in pure phospholipid vesicles (Sundler et al., 1978; Wilschut et al., 1979). Hemolysis of fresh human red blood cells is not induced to any significant extent by bee venom PLA_2, even though almost all the phospholipids in the outer leaflet of the membrane bilayer have been hydrolyzed (Zwaal et al., 1975; Fletcher et al., 1987).

Louw and Visser (1978) reported that some CTX fractions were contaminated with trace amounts of venom PLA_2, which greatly potentiated the hemolytic activity of the CTX protein. Trace contamination of crude CTX preparations with PLA_2 activity has confounded the interpretation of some of the toxic actions of CTXs, especially when tested on hemolysis of erythrocytes (Harvey, 1985; Louw and Visser, 1978; Harvey et al., 1983). Highly purified CTXs are considered virtually devoid of PLA_2 contamination and have considerably reduced hemolytic potency compared to PLA_2 contaminated preparations. However, the potency of CTX on other preparations, such as skeletal muscle, is unaffected by PLA_2 contamination. Therefore, PLA_2 activity has been considered to be essential for the hemolytic action of CTXs on erythrocytes, suggesting the mechanism of action of CTX on the red blood cell does not reflect the same mechanism in skeletal muscle (Harvey, 1985). However, more recent studies have demonstrated that higher concentrations of PLA_2 do act in synergy with CTX in skeletal muscle and that the apparent difference in action of the CTXs may be related to differences in the concentration and type of free fatty acids in the two tissues in the absence of CTX (Fletcher and Lizzo, 1987), not differences in mode of CTX action.

One problem with determining the role of PLA_2 activity in the action of CTXs is that most investigators analyze PLA_2 contamination of CTX preparations by measuring the enzymatic activity on purified phospholipid or egg yolk substrates by various titration assays. Titration assays measure fatty acid release and cannot distinguish between PLA_1 (EC 3.1.1.32; removal of fatty acid from #1 position) and PLA_2 (removal of fatty acid from #2 position) activities. PLA_1 and PLA_2 activities are collectively referred to in the present study as PLA activity. The results of titration assays are considered to accurately reflect PLA_2 activity in venom research, as PLA_1 activity is not present at detectable levels in snake venoms (Rosenberg, 1979). CTX preparations that have no PLA activity on artificial substrates have been assumed to be enzymatically inactive on biological membrane systems such as

the red blood cell. Rosenberg (1979) has cautioned against such assumptions about PLA_2 activity and has suggested that PLA_2 activity must be directly determined on the biological substrate that is used for pharmacological or toxicological studies.

Snake venom CTX shares many of the properties of bee venom melittin, including interactions with PLA_2. The action of melittin on biological membranes has been better characterized than that of CTX, therefore melittin serves as a good model for CTX studies. Melittin, a low molecular weight (2,840) polypeptide of 26 amino acids, comprises about 50% of the dry weight of bee venom (Habermann, 1972). Among its toxic actions, melittin causes hemolysis of red blood cells and is cytolytic to other cell types (Habermann, 1972). Melittin enhances the rate of bee venom PLA_2 activity 5- to 6-fold on sonicated (Mollay and Kreil, 1974) and up to 300-fold on nonsonicated (Yunes et al., 1977) liposomes. Similar studies have not been conducted with CTX, despite its reported interaction with PLA_2. Understanding the mechanism by which melittin enhances bee venom PLA_2 activity is important, as melittin is used by investigators as a probe to activate tissue PLA_2 activity (Mollay et al., 1976; Shier, 1979). The use of melittin as a tissue PLA_2 activator evolved from the initial observations with bee venom and presumes some specificity to this action. Recently investigators have proposed that melittin enhances the rate of bee venom PLA_2 activity on multilamellar vesicles (nonsonicated liposomes) by converting these concentric bilayers into large unilamellar vesicles and thereby exposing a greater phospholipid surface to the enzyme (Dufourcq et al., 1986). However, these studies did not actually examine the interaction between melittin and PLA_2 and were conducted in the absence of Ca^{2+}, which, in addition to supporting PLA_2 activity, normally binds to the headgroup of phospholipids and stabilizes membranes. The absence of this divalent cation could have contributed to the destabilizing effect of melittin on the multilamellar vesicles. Also, the conversion of multilamellar vesicles to large unilamellar vesicles does not explain the enhancement of bee venom PLA_2 on unilamellar substrates (sonicated liposomes) by melittin (Mollay and Kreil, 1974). However, melittin appears to cause local perturbations of bilayers (Dufourcq et al., 1986) that may somehow increase the access of PLA_2 to the phospholipid substrate. As with CTX, it is difficult to isolate melittin fractions that are free from contamination with venom PLA_2 activity (Mollay et al., 1976).

The relationship between the potency of CTX and the lipid composition of the target membranes is unclear (Condrea, 1979). It has been suggested that tissues with higher levels of free fatty acids are less sensitive to the interaction between CTX and PLA_2 (Fletcher and Lizzo, 1987). The relationship between free fatty acids and the response of membranes to CTX alone has not been directly examined.

Internalization is required for the action of a number of bacterial toxins (Middlebrook and Dorland, 1984). The rapid time to onset of effect (seconds; Fletcher and Lizzo, 1987) suggests signal transduction is a more likely mechanism for CTX action. Regardless the actual location of the toxin molecule (inside or outside of the cell), it is important in developing prophylactic and therapeutic measures to determine accessibility of antibodies to the toxin site for neutralization.

Rationale:

Several model systems were used to examine: (1) the mode of action of snake venom CTXs; (2) the dependence of this action on the membrane lipid composition; (3) the interaction of CTX with PLA activity; (4) the effects of membrane composition on CTX action; (5) internalization of CTX, and; (6) the similarities and differences between CTXs and the presynaptically-acting snake venom PLA_2s. The advantages and disadvantages of each system are:

1. Skeletal Muscle. Contractures of skeletal muscle are believed to reflect the "true" cardiotoxic mechanism of CTXs. A special advantage of this preparation is that we have access to human skeletal muscle, which differs from the commonly used rat diaphragm preparation in Ca^{2+} dependence of CTX action (Fletcher and Lizzo, 1987). Disadvantages of skeletal muscle are the great degree of biological variability involved in examining contracture induction, the small number of studies that can be run in a single day and the inability to conveniently use fluorescence probes to directly monitor myoplasmic Ca^{2+} concentrations or patch-clamp electrophysiology to directly examine ion currents.
2. Erythrocytes. Despite some disagreement (Harvey, 1985), the action on red blood cells may reflect the "true" cardiotoxic mechanism of CTXs (Fletcher and Lizzo, 1987). The red blood cell has many advantages, including: it is a simple model system in which all the lipids are contained in the plasma membrane (no organelles in erythrocytes); red blood cells of different lipid composition can be obtained from a wide variety of species (we have contacts with University of Pennsylvania School of Veterinary Medicine and the Philadelphia Zoo); the hemolysis assays with red blood cells allow many experiments to be conducted in a single day on the same batch of cells and they are far less subject to biological variability than skeletal muscle. The disadvantages of RBCs are the limited number of toxins that are active on them and their paucity of responses that can be examined, as apposed to excitable cells (muscle and nerve).
3. Lymphocytes. We have recently demonstrated lymphocytes to be a target for CTX action. Lymphocytes, like red blood cells, can be obtained from a large number of species. Patch-clamp electrophysiology for monitoring plasmalemmal ion currents and direct monitoring of cytoplasmic Ca^{2+} can be done with lymphocytes. Lymphocytes can be preloaded overnight with radiolabeled lipid precursors and subtle aspects of lipid metabolism examined the following day. Disadvantages of lymphocytes include their lack of availability (they must be isolated rapidly from fresh blood - free donors are scare) and limited number of ion currents (primarily K^+).
4. Platelets. Platelets are a rich source of tissue PLA_2 activity. Human platelets are readily available from the Red Cross.
5. Synaptosomes. Synaptosomes, or other nerve preparations, are essential for studies on presynaptically acting PLA_2 neurotoxins, as these toxins are specific for nerve tissue. Synaptosomes have the advantages that that they provide a relatively large tissue mass for biochemical studies of presynaptic terminals impossible to obtain with phrenic nerve-diaphragm preparations, intrasynaptosomal Ca^{2+} can be monitored with fluorescent dyes and acetylcholine release can be monitored with radiolabeled choline. The major disadvantage of synaptosomes is that they are not currently a good model for PLA_2 toxin studies. The reasons that they are

not appropriate are: they have greatly elevated fatty acid levels that may mask subtle effect of PLA_2 toxins, and; the optimum conditions for simulating the action of the toxins at the neuromuscular junction have not been satisfactorily worked out. We are attempting to correct these problems.

6. Cell Lines. Cell lines provide homogeneous biological material. They can be rich in ion currents, depending on the cell type. The epithelial cells, like lymphocytes, have an advantage over muscle in that intracellular cytosolic Ca^{2+} concentrations can be monitored directly with fluorescence probes, such as indo-1. Additionally, patch-clamp electrophysiological studies can be conducted in which the effect of the toxin on specific membrane currents can be directly examined. Skeletal muscle patch-clamp studies require the use of cell culture, which might suppress expression of toxin sensitive channels (appears to be the case in cultured human and equine skeletal muscle; unpublished observations). The advantage in using both cell types is that epithelial cells have Ca^{2+} and voltage activated K^+ channels and lymphocytes have K^+ channels inhibited by Ca^{2+}. Additionally, cell lines can be used for detailed anaysis of lipid metabolism, as the phospholipid and neutral lipid pools can be readily radiolabeled overnight. Cell lines aloow extensive lipid metabolism studies as they can be readily radiolabeled. The major disadvantages of cell lines are the time spent cloning and maintaining the cells and the "abnormal" responses that may be induced by either culture conditions or the process of immortalizing the cells.
7. Primary Cell Cultures. Primary cultures allow a far greater number of different patients and animals to be examined than cell lines.
8. Artificial Membranes. Artificial membranes can be prepared in many different forms, all of which yield different types of information regarding membrane perturbing agents. The membranes can be: mixed micelles, which are monolayers of phospholipid and a detergent (usually Triton X-100); unilamellar vesicles, which are bilayers of either a single phospholipid or mixture of phospholipids, and; multilamellar vesicles, which are concentric bilayers of phospholipids. These different membranes, all having different amounts of phospholipid exposed to the incubation medium, can be used to examine substrate availability and membrane penetration in PLA_2 studies. The primary disadvantage of these preparations is their dissimilarity in behavior to more complex biological membranes.

The role of PLA_2 activity in the mode of action of the toxins can be examined by removing Ca^{2+} from the incubation medium, or by replacing Ca^{2+} with Sr^{2+}. This approach is applied in the current Annual Report to the hemolytic action of the CTXs.

The effects of the toxin on lipid metabolism of the red blood cell, primary cell cultures and cell lines can be directly examined using gas chromatographic analysis of free fatty acids or the metabolism of radiolabeled phospholipids. Highly detailed studies of effects on endogenous lipid metabolism can be conducted in primary cell cultures and cell lines with neutral and phospholipids preradiolabeled by feeding the cells ^{14}C-fatty acids overnight.

Internalization of the toxin can be determined in a number of ways. Our initial studies examined the patterns of phospholipid hydrolysis and free

fatty acid release. The studies in the current Annual Report examined the internalization of PLA_2 by the CTXs and melittin, as determined on artificial bilayers.

The synergistic interaction between CTX and PLA_2 can be examined using artificial substrates. Knowing that about 70% of the phospholipid is in the outer layer of liposomes, monitoring the extent of phospholipid hydrolysis can provide information on the ability of the PLA_2 to penetrate bilayers.

β-bungarotoxin (β-BTX) and other presynaptically-acting snake venom PLA_2s exhibit a triphasic action on acetylcholine release from the phrenic nerve-diaphragm preparation (Chang, 1979). The observation of these phenomena is based on electrophysiological studies of events (EPPs, MEPPs) having durations of milliseconds. The relative insensitivity of even the most sensitive biochemical assays for acetylcholine requires that the transmitter be collected over periods of seconds to minutes. Therefore, extensive analysis of the time and concentration dependence of β-BTX action must be done in order to recreate in this biochemical model the same effects observed in the electrophysiological model. We have previously developed a crude method with which the second and third phases of the triphasic effect can be emulated in this biochemical model (Fletcher and Middlebrook, 1986). However, the model has to be refined, including application to a more highly purified synaptosomal fraction and improvment of the methods of isolation to reduce the extensive lipolysis that occurs during homogenization. Once this model has been sufficiently developed, then it can be applied to studies comparing the mechanisms of CTX action to those of the PLA_2 neurotoxins.

Experimental Methods:

Materials. Venom from *Naja naja atra*, CTX from *Naja naja kaouthia* venom (Lot# 125F-4007), bee venom PLA_2 (*Apis mellifera*), melittin, β-bungarotoxin, Tris base, Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), Mes (4-morpholineethanesulfonic acid), bovine serum albumin (fatty acid free), 2'-7'-dichlorofluorescein, free fatty acid (methylated and unmethylated) standards, phospholipid, triglyceride and cholestero' standards, and butylated hydroxytoluene were purchased from Sigma Chemical Company (St. Louis, MO). CTX from *Naja naja atra* venom was purified from venom by ionic exchange chromatography, as described in the first Annual Report. The *N. n. kaouthia* and *N. n. atra* CTX fractions each appeared on SDS-PAGE slab gels as a single protein band of about 7,000 MW. The PLA_2 from *N. n. atra* venom was provided by Dr. Leonard Smith.

Hemolysis of Erythrocytes. Erythrocytes were obtained from the American Red Cross Blood Services and stored as CPD whole blood (AS-1) at 4°C. These cells were used within about two weeks (plus or minus) of their expiration date.

Before incubation with toxin erythrocytes were washed three times in HEPES buffer (HEPES 20 mM, NaCl 130 mM, room temperature, pH adjusted to 7.4). Following the final centrifugation step 4 µl aliquots of packed red blood cells were added to 0.6 ml of the incubation medium (HEPES buffer with indicated pH, temperature, concentration of divalent cation and glucose). The preparations were incubated for 2 hr with or without toxin, centrifuged and hemoglobin release estimated by reading the absorbance of the supernatant at 540 nm. The 100% hemolysis point was determined by incubating 4 µl of erythrocytes in 0.6 ml of distilled water. Blanks containing no CTX were

subtracted from all samples.

Treatment of Toxin Fractions With p-Bromophenacyl Bromide. Irreversible inactivation of PLA_2 activity was done by the method of Pieterson et al. (1974). Bee venom PLA_2 and *N. n. kaouthia* CTXs (30 µM) were incubated for 0 or 20 hr at 37°C in 600 µl of sodium cacodylate buffer (0.1 M) containing NaCl (0.1 M), p-bromophenacyl bromide (300 µM; added from 10 mM stock made up in acetone) and adjusted to pH 6.0. Following the reaction with p-bromophenacyl bromide, PLA_2 activity and hemolytic activity were determined. A mixed micelle substrate comprised of phosphatidylcholine from egg yolk (5 mM) and Triton X-100 (10 mM) was used to determine PLA_2 activity. The phospholipid was evaporated to dryness under N_2. Subsequently the detergent and 1 ml buffer [Tris (100 mM), bovine serum albumin (0.5%), and Ca^{2+} (2 mM), pH adjusted to 7.4] were added and the preparations were sonicated until clear. Treated PLA_2 or CTX (CTXs were frozen and lyophilized first to concentrate the toxin) was added to the substrate buffer and the samples were incubated for 30 min at 37°C. The incubation was terminated by addition of extraction mixture and the free fatty acids were then titrated (Dole, 1956). Hemolytic activity was tested as described above using a 60 µl aliquot of the p-bromophenacyl bromide-treated toxin (3 µM final toxin concentration) and adjusting the hemolysis HEPES buffer (HEPES 20 mM, NaCl 130 mM, Ca^{2+} 2 mM) to pH 9.0 prior to addition of the toxin. The final pH of the actual incubation medium after mixing the cacodylate and HEPES buffers was 7.8.

Preparation of Mixed Micelle, Unilamellar and Multilamellar Substrates. Mixed micelles were prepared by evaporating the appropriate phospholipid substrate to dryness under N_2 and adding Triton X-100 at the indicated ratio to PC. Tris buffer (Tris 100 mM; Ca^{2+} 2 mM; BSA, 0.5%, pH 8.0) or HEPES buffer (HEPES 20 mM, Ca^{2+} 2 mM, BSA 0.5%, pH 7.4) was added and the preparations were sonicated until clear. Radiolabeled substrate, L-3-PC (1-stearoyl-2-[1-^{14}C]arachidonyl), was added to this latter buffer at a concentration of 10 µM. Multilamellar vesicles were prepared by lyophilizing egg yolk PC overnight to remove all organic solvents. Tris buffer was added and the phospholipid (1 mM final concentration) was allowed to swell at room temperature for 2 hrs. Small unilamellar vesicles were prepared in the same manner as the multilamellar vesicles, except that the preparations were sonicated for 20 min before use.

Gas Chromatographic Analysis of Fatty Acids. Lipids were extracted from blood cells (Fletcher et al., 1987; Fletcher et al., 1990b) and the free fatty acids separated and analyzed by gas chromatography as their methyl esters, as previously described (Fletcher et al., 1987; 1988; 1989; 1990b; 1990c).

Determination of Phospholipase A_2 Activity. The substrates above were incubated with or without PLA_2, CTX or melittin for the indicated time and temperature. The incubation was terminated by extracting and titrating the free fatty acids (Dole, 1956), or, when using radiolabeled substrates, the incubates (1 ml) were extracted with 2 ml CH_3OH, and, after 30 min, 2 ml $CHCl_3$ and 1.5 ml H_2O were added. The lower phase (chloroform) was removed and evaporated under N_2 and brought up in 200 µl of $CHCl_3$ for spotting on silica gel plates. The neutral lipids were separated by 1-D TLC, as previously described (Fletcher et al., 1987). The plates were dried and the lanes scanned for radioactivity with a Raytest (McMurray, PA) RITA Radio TLC Imaging Analyzer (Fletcher et al., 1990a; 1990d). Free fatty acids were identified by comparing R_f values with radiolabeled standards developed in separate lanes on each TLC plate.

Radiolabeling Lipid Fractions in Skeletal Muscle Primary Cultures.

Primary cultures of human and equine skeletal muscle will be derived and maintained as previously described (Wieland et al., 1989). Cells are radiolabeled with ^{14}C-linoleic acid for 3 days, as previously described for airway epithelial cell lines (Fletcher et al., 1990a; 1990d). The lipids are extracted, separated by 1-D TLC and radioactivity determined by scanning the plate, as previously described (Fletcher et al., 1990a; 1990d).

Preparation of Synaptosomes and Determination of Acetylcholine (ACh Release) and Choline Uptake. Studies were done using the crude P_2 pellet preparation, as previously described (Fletcher and Middlebrook, 1986), or the synaptosomal preparation described by Dunkley et al. (1988). Briefly, whole brain from rat or mouse is homogenized (1 g tissue per 9 ml) in a Teflon-glass homogenizer containing sucrose (0.32 M), EDTA (1 mM), dithiothreitol (0.25 mM), pH 7.4 (4°C). The homogenate is centrifuged (1,000 x g; 10 min) and the supernatant adjusted to 14 ml for a protein concentration of 5 mg/ml. The supernatant is applied to a discontinuous Percoll gradient (2 ml each 23%, 15%, 10%, 3% Percoll; v/v; pH 7.4) and centrifuged at 32,500 x g for 5 min. Fractions 3 and 4 will be used in our studies. Incubation of the synaptosomes with radiolabeled choline and β-BTX, centrifugation (microcentrifuge; 4°C) of the samples and removal of the supernatants is similar to previous studies (Fletcher and Middlebrook, 1986). ACh release is determined by lyophilizing the supernatant overnight. The sediment is resuspended in methanol (50 µl), spotted on TLC plates, developed in one dimension with n-butanol:methanol:acetic acid:ethylacetate:water (4:2:1:4:3; Bluth et al., 1980) and the radioactivity corresponding to ACh and choline analyzed by an imaging scanner. Choline uptake was performed basically as previously described (Fletcher and Middlebrook, 1986), except that the synaptosomes were filtered (Whatman GF/B filters) to terminate uptake in a Hoefer FH224V filter holder and washed twice with 10 ml cold (4°C) buffer. Scinti Verse™ II (Fisher Scientific Co.) was used for liquid scintillation counting.

Results:

PROBLEM 1. What are the effects of CTX alone and CTX-PLA_2 combinations on contracture induction of human and rat skeletal muscle and on Ca^{2+} transients in human and equine lymphocytes?

No studies specifically related to this problem were conducted this year.

PROBLEM 2. What is the dependence of CTX action on fatty acid distribution and the free fatty acid content of muscle and red blood cells?

No studies specifically related to this problem were conducted this year.

PROBLEM 3. What are the hemolytic effects of CTX alone and CTX-PLA_2 combinations in red blood cells from different species?

The effects of prior p-BPB treatment and the presence of Ca^{2+}, Sr^{2+} or Mn^{2+} in the incubation medium on the hemolytic action of *N. n. kaouthia* CTX has previously been reported. We had assumed that the effect of all these conditions would be the same for the *N. n. atra* CTX. However, repeating this analysis with a much higher volume of packed red blood cells (to be compatible with the fatty acid analysis described below), we reproduced the same effects

with the *N. n. kaouthia* CTX, but found that the *N. n. atra* CTX did not have the Sr^{2+}-supported hemolytic activity observed with the *N. n. kaouthia* CTX (Figure 1). The Mn^{2+} blank was high (about 4-5x normal) in these experiments for reasons apparently related to the large volume of cells used (data not shown). The entire hemolytic activity of the *N. n. atra* CTX that was independent of PLA_2 activity was observed in a Mn^{2+}-containing medium, as judged by the similarities between p-BPB-treated and nonp-BPB-treated CTXs in hemolytic activity in Sr^{2+} medium (Figure 1). In contrast, the *N. n. kaouthia* CTX had a Sr^{2+}-supported hemolytic activity evident even after p-BPB treatment. Notice that the *N. n. atra* CTX has a much greater PLA_2-dependent component than does the *N. n. kaouthia* CTX (Figure 1).

PROBLEM 4. What are the effects of CTX on endogenous lipolytic enzymes in skeletal muscle and red blood cells?

In addition to the studies on CTX, we have been conducting studies on another cardiotoxin-like peptide (melittin) from bee venom. Melittin has been reported to activate bee venom (Mollay and Kreil, 1974) or endogenous (Mollay et al., 1976) PLA_2 activity. We had demonstrated in a previous quarterly report that cardiotoxin fractions "appear" to activate endogenous PLA_2, but in reality are contaminated with trace amounts of PLA_2 activity. Some inconsistencies have been observed with melittin as an activator of endogenous PLA_2 (Metz, 1986; Rosenthal and Jones, 1988). These reports prompted us to reexamine melittin and CTX to determine if the alleged "activation of endogenous PLA2" is due to contamination of the toxin fractions with their respective venom PLA_2s. Recently we have directly examined the effects of melittin on endogenous PLA_2 activity in a more direct manner than other investigators. Since melittin and CTX potentiate PLA_2 activity, it makes little sense to compare the effects of PLA_2 in the absence of melittin or CTX to those of melittin or CTX containing PLA_2 contamination. As previously reported, melittin fractions can be treated with p-bromophenacyl bromide (p-BPB) (melittin has no histidine groups to react with p-BPB) to inhibit PLA_2 activity contaminating the fraction without affecting the activity of the melittin molecule.

Effects of melittin on endogenous PLA_2 activity in airway epithelial cells

Three different cell lines were radiolabeled with ^{14}C-fatty acid prior to beginning the experiments (Table 1; Fletcher et al., 1990d). There was a slight difference in labeling for the different cell lines in the absence of toxin that was not of any consequence to the outcome of this specific study (Table 1). However, since one of the cell lines was from a cystic fibrosis patient (CFNP1), these studies may have some significance in understanding lipid metabolism in this disorder *and* in better understanding the toxin action. Bee venom PLA_2 caused a significant decrease in total phospholipid corresponding to the increased free fatty acid (Table 2). The native melittin fraction caused greater hydrolysis of phospholipids than did bee venom PLA_2 (Table 2). The predominant phospholipid hydrolyzed by addition of the melittin fraction was phosphatidylcholine (Table 3). There were no significant effects of either PLA_2 or melittin on the diglyceride or triglyceride fractions (Table 2). However, the triglyceride fraction was not highly labeled in these cell lines. The percentage of bee venom PLA_2 contamination in the melittin fraction

was estimated to be about 0.1% based on previous studies. Treatment of melittin fractions with p-BPB decreases the PLA_2 activity of these fractions by greater than 95%, as determined using radiolabeled substates. Pretreatment of the melittin fraction with p-BPB for 20 hrs at 37°C greatly reduced the PLA_2 activity, relative to the native toxin, when the toxin was subsequently added to the cells, as judged by increased label associated with total phospholipid, decreased (75%) free fatty acid (Table 2) and decreased hydrolysis of phosphatidylcholine (Table 3). The free fatty acid values for the p-BPB-treated melittin fraction are not significantly different from the control values by the Newman-Keuls test. However, the individual values for the control preparations (0.8%, 1.3%, 3.4%, respectively, for HUNNNMAS-301, BEAS-2 and HUCFNPAS-101) compared to those for the p-BPB treated preparations (4.4%, 10%, 15%, respectively), suggest some residual PLA_2 activity may remain associated with this fraction. When bee venom PLA_2 is added to the p-BPB-treated fraction at an amount approximately equal to the estimated contamination in the native melittin fraction, phospholipid hydolysis returns to the levels of the native melittin fraction. The native melittin fraction and p-BPB-treated melittin plus PLA_2 treatments result in identical values for the decrease in phospholipid, increase in free fatty acid (Table 2) and the hydrolysis of phosphatidylcholine (Table 3).

Effects of CTX on endogenous PLA_2 and other lipolytic activities in primary cultures of skeletal muscle

We tested the uptake of four different fatty acids (18:0; 18:1; 18:2 and; 18:3) in primary muscle cell cultures. Human primary muscle cell cultures were radiolabeled with ^{14}C-fatty acid for 24, 48 or 72 hrs (Table 4). The nonessential fatty acids (18:0 and 18:1) appeared to be taken up less preferentially (18:0), and/or undergo considerable metabolism and loss of total radiolabel (18:0; 18:1). We verified that the specific activities of the stock fatty acids were almost identical to rule out differences in specific activities as accounting for the lower counts for 18:0 and 18:1. We decided to continue to radiolabel cells with 18:2, as this fatty acid is normally abundant in cell phospholipid and neutral lipid fractions, unlike the low levels of 18:3 (see Fletcher et al., 1988) and does not appear to undergo extensive metabolism (Table 4). In general, we have found that "older cultures" (those plated for 3-4 wks) exhibit much greater radiolabeling of cardiolipin

Human primary muscle cell cultures were radiolabeled with ^{14}C-fatty acid prior to beginning the experiments (Table 5). In the first culture tested CTX (10 μM; 2 hrs) caused a specific decrease in cardiolipin (plus a slight decrease in total phospholipid) in parallel with an increase in diglyceride and free fatty acid. Also, there appeared to be a decrease in cholesterol esters. Both of these actions (cardiolipin and cholesterol ester hydrolysis) would produce free fatty acids. In addition, hydrolysis of cardiolipin produces diglycerides. Pretreatment of the CTX fraction with p-BPB for 20 hrs at 37°C greatly reduced the PLA_2 activity due to snake venom PLA_2 contamination, relative to the native toxin (unpublished observations). Addition of p-BPB-treated CTX to the cells did not alter the patterns of lipid distribution relative to the native CTX. This observation, plus the lack of evidence for hydrolysis of any phospholipid except cardiolipin (not by a PLA_2 mechanism), suggests that PLA_2 contamination in this highly purified CTX fraction is insignificant and plays no role in the action of highly purified

toxin on biological substrates.

In cultures 1 wk old CTX clearly caused a time-dependent decrease in cholesterol esters and triglyceride in parallel with an increase in diglyceride and free fatty acid (Table 6). We examined shorter incubation times with toxin (5-60 min) than those in the above study (2 hrs). In contrast to our previous study, cardiolipin was not hydrolyzed to any great extent in the first study of the present quarter (Table 6). Consistent with specific hydrolysis of these neutral lipids, the phospholipid fraction remained about 70% of the total radiolabel (Table 6). Note that the cardiolipin fraction was not well labeled in Table 2. We attributed this to the age of the culture.

We repeated this study with an older cell culture (2 wks after plating) and verified that cardiolipin is better labeled in aged cultures (Table 7). Cardiolipin was only slightly, if at all, decreased by CTX. Therefore, the effect of CTX on cardiolipin metabolism appears to require a longer time than the 1 hr period used in the present study. In agreement, relatively long incubation periods are required for hemolysis of red blood cells (see first Annual Report). The major effect of p-BPB treatment was to decrease triglyceride levels relative to those observed with the native toxin (Table 7). It is possible that the triglyceride and cholesterol ester levels are high relative to the native toxin due to elevated PLA_2 activity (venom enzyme contamination) in the native fraction. The free fatty acids would be diverted to the pool of triglycerides. In support of this hypothesis, phospholipid levels are lower and free fatty acid values are higher with the native toxin relative to the p-BPB-treated toxin (Table 7).

A number of factors affecting the studies of CTX action have been identified. These include: 1) the type of fatty acid used to label the cells (prefer 18:2); 2) the age of the cell culture (2 wks better than 1 wk for labeling cardiolipin and cholesterol esters); 3) the time of exposure to CTX (the longer the better); 4) the use of highly purified and p-BPB-treated fractions. Future studies examining CTX action will involve longer incubation times (60-120 min) with the 3 μM concentration of CTX and higher concentrations (ca. 10 μM) of toxin at these same times. "Older" (ca. 2 wk) cultures will be used to optimize labeling of cardiolipin phosphatidic acid and cholesterol esters with 18:2.

We have found that more consistent results in cell cultures of skeletal muscle are obtained with a concentration of 10 μM *Naja naja kaouthia* CTX and a 2 hr incubation period. Both the native and p-bromophenacyl bromide (p-BPB)-treated *Naja naja kaouthia* snake venom CTXs cause the production of diacylglyceride and free fatty acid in primary cultures of skeletal muscle from normal or malignant hyperthermia susceptible patients (Table 8). This table demonstrates the typical variation between 35 mm culture dishes from the same patient. Note that the normal patients also exhibit a decrease in phospholipid labeling; whereas the malignant hyperthermia patients exhibit a decrease in triglyceride labeling (Table 8). The effects on triglyceride metabolism appear to relate specifically to the malignant hyperthermia disorder and not to the relatively higher level of radiolabeling of triglycerides in the malignant hyperthermia patients. For example, primary cultures from a normal patient with high triglyceride labeling do not exhibit a significant decrease in triglyceride radiolabel with toxin treatment (Table 9). The importance of reducing the venom PLA_2 contamination in the CTX fraction can readily be seen with the less highly purified CTX from *Naja naja*

atra snake venom (Table 10). Notice that p-BPB treatment of the fraction dramatically reduced PLA_2 activity. Comparing larger patient populations (rather than the individual patients in Table 8), the relationship between decreased phospholipid and the effects of CTX on normal cells is maintained (Table 11). Also, *all* cells treated with the toxin (native or p-BPB-treated) exhibit increased diglyceride and free fatty acids (Table 11). Cells from the two malignant hyperthermia patients both exhibited decreased triglyceride (Table 11). Also, the malignant hyperthermia cells treated with the toxin (native or p-BPB-treated) exhibit increased diglyceride and free fatty acids (Table 11). In general, normal equine muscle cells are similar to normal human cells in response to the CTX fractions (Table 12). In contrast, cells from a horse with hyperkalemic periodic paralysis did not exhibit the production of diglyceride (Table 12), but did exhibit the increase in free fatty acids. While the results are rather preliminary, it appears that melittin has a slightly different effect on normal muscle than does snake venom CTX (Table 13). First, the triglyceride decrease seen only in malignant hyperthermia muscle with CTX is seen in normal muscle with melittin (Table 13). Next the ratio of free fatty acid production to diglyceride production by melittin is greater in muscle from malignant hyperthermia patients than normals (Table 13). There is also a selective hydrolysis of phosphatidylcholine by the Sigma fraction and large decrease in phospholipid labeling due to the PLA_2 contamination in this fraction (Table 13; Fletcher et al., 1990).

Effects of CTX on hydrolysis of red blood cell phospholipids

We examined the hydrolysis of red blood cell phospholipids by fractions of bee venom PLA_2 ($BVPLA_2$), snake venom (*N. n. atra*) PLA_2 ($SVPLA_2$), *N. n. kaouthia* CTX (NnkCTX), *N. n. atra* CTX (NnaCTX) and p-BPB-treated derivatives of the CTXs (NnkCTXB, NnaCTXB). The PLA_2s and untreated CTX fractions (especially that from *N. n. atra* venom) caused extensive hydrolysis of phospholipids, as evidenced by very high levels of fatty acids relative to preparations not exposed to toxin (Table 14). Inactivation of the PLA_2 contamination in the CTX fractions by treatment with p-BPB totally abolishes any PLA_2 activity on red blood cells, suggesting either: (1) CTX does not activate endogenous PLA_2 enzymes, or (2) there is no PLA_2 enzyme in red blood cells for the toxin to activate.

Effects of melittin on bee venom PLA_2 activity

All of the studies in the present report were done with p-BPB-treated melittin. The p-BPB-treated melittin contains about 1.2% of the PLA_2 contamination contained in the native fraction (unpublished observations - determined on mixed micelle substrates). We examined the effects of melittin on an arachidonic acid containing substrate embedded in an egg yolk PC:Triton X-100 matrix, or in MLVs or ULVs. Radiolabeled L-3-PC (1-stearoyl-2-[1-^{14}C]arachidonyl) was used to determine the level of hydrolysis in all cases. The incubates were extracted and radioactivity in the neutral lipids analyzed by radioimaging scanning of TLC plates. We first examined the effects of PLA_2 concentration on the Ca^{2+} and NaCl-dependent melittin stimulation of the hydrolysis of mixed micelles. There was no stimulation of PLA_2 activity by melittin in the presence of Ca^{2+} alone (i.e., in the absence of NaCl), despite a range of phospholipid hydrolysis values in the absence of melittin of about 5 to 45% (Figures 2 and 3). Note that each figure is a separate set of experiments and that these experiments are done on separate days. In contrast,

melittin did stimulate bee venom PLA_2 activity on mixed micelles under the following conditions (Figures 3 and 4): 1) a NaCl concentration of 130 mM; 2) a PLA_2 concentration > 5 nM (phospholipid hydrolysis > 15%) and; 3) a concentration of melittin of 10 μM.

We assumed that the increase in amount of hydrolysis on ULVs and MLVs with increasing concentrations of PLA_2 was due to one of four possibilities: 1) PLA_2 enzymes can penetrate bilayers, despite a large body of literature indicating otherwise; 2) incomplete hydrolysis had occurred (not the maximum extent) due to too short an incubation time; 3) the PLA_2 fractions may contain trace contamination with melittin; and 4) nonproductive binding occurs that inactivates the enzyme. We examined the consequence of an increased incubation time on bee venom PLA_2 activity on a ULV substrate. The activity appeared to have already reached a plateau at 2 hr *for a fixed concentration of PLA_2*, suggesting that the PLA_2 enzyme does not penetrate the membrane bilayer and that we were not dealing with incomplete hydrolysis (Figure 4). However, the amount of hydrolysis increased with increasing enzyme concentration (Figures 4A and 4B), defying rules of normal enzyme kinetics. We conclude that trace melittin contamination in the bee venom PLA_2 fraction would be insufficient to allow the enzyme to penetrate the bilayer, based on the high concentration of melittin relative to PLA_2 required for increased hydrolysis (Figure 5 and 6). The results obtained with ULVs and radiolabeled substrates consistently demonstrate the enhancement of PLA_2 activity by melittin under every condition examined (e.g. Figures 5 and 7). Our previous studies with MLVs suggested that Ca^{2+} was essential for melittin to increase the maximum extent of substrate hydrolysis by PLA_2, but only at low (30 nM) concentrations of PLA_2. We repeated these studies and found that Ca^{2+} was not essential for this action of melittin (Figure 7). It is possible that at low levels of hydrolysis the effects of melittin are less obvious, as demonstrated on mixed micelles (Figure 3) and that this may account for our previous negative findings.

In examining the time course of PLA_2 activity on ULVs, we observed that stopping the reaction with methanol and allowing the samples to stand for up to 3 hrs before completing the extraction steps (commonly used approach) resulted in variable results, with greater levels of hydrolysis associated with shorter incubation times (Table 15). We attribute this to methanolysis of the phospholipids - replacing the fatty esters with methyl groups. An even less satisfactory condition was stopping the reaction with EGTA (used by many investigators), which resulted in *reduced* levels of hydrolysis in the early time points (Table 15). We have no explanation for this adverse effect of EGTA. The most consistent results were obtained when the specimens were immediately extracted completely (Table 15).

Effects of snake venom CTX on bee venom and snake venom PLA_2 activities and of melittin on snake venom PLA_2 activity

We tested whether CTX could allow bee venom PLA_2 to penetrate the bilayer and whether CTX had any stimulatory activity on bee venom PLA_2 activity (Figure 8). At low concentrations of bee venom PLA_2 ($\leq$10 nM), CTX greatly increased the enzymatic activity of the bee venom PLA_2. However, at higher concentrations of PLA_2 ($\geq$100 nM), CTX had no effect on phospholipid hydrolysis. There was no apparent CTX-facilitated penetration of the membrane bilayer at high concentrations of the bee venom enzyme (Figure 8), in agreement with melittin (Figure 6).

We compared the maximum extent of phospholipid hydrolysis by *Naja naja atra* PLA_2 to that of bee venom PLA_2 and found that the concentration of the snake venom enzyme had to be about 10-fold greater than the bee venom enzyme for hydrolysis of the entire outer leaflet of the membrane bilayer (compare Tables 16 and 17 to Figure 4). Next we compared the effects of snake venom CTX and bee venom melittin on the snake venom PLA_2 activity (Figure 9). CTX had very little effect on high concentrations of snake venom PLA_2 (Figure 9A), in agreement with bee venom PLA_2 (Figure 8). The maximum extent of hydrolysis was less for the snake venom PLA_2 in the presence of CTX (Figure 9A), than in the presence of melittin (Figure 9B). Examining the effects of NaCl and Ca^{2+} on the interaction between snake venom PLA_2 and CTX revealed that these cations increase the maximum extent of hydrolysis (Figure 10). In the presence of Ca^{2+} and/or NaCl, CTX did not further enhance the hydrolysis of ULVs by the snake venom enzyme (Figure 10). We conclude that at high concentrations of PLA_2, CTX only seems to replace cations.

The PLA_2 from *Naja naja atra* snake venom exhibits the same concentration-dependent degree of hydrolysis we previously observed for bee venom PLA_2 (Tables 16 and 17). At a concentration of 3 μM, bee venom PLA_2 is capable of hydrolyzing the ULVs to a greater extent than snake venom PLA_2 (Table 16). Unlike bee venom PLA_2, which was unaffected by BSA (Figure 11), snake venom PLA_2 activity on ULVs is greatly enhanced by the presence of 0.5% BSA in the incubation medium (Table 18). We previously reported that the interaction of melittin with bee venom PLA_2 was highly dependent on NaCl in the medium and independent of Ca^{2+}. The opposite is true for snake venom PLA_2 and CTX (Table 19). In the absence of BSA, NaCl antagonized the enhancement of snake venom PLA_2 activity by *Naja naja kaouthia* CTX (Table 19). The presence of Ca^{2+} overcame the inhibitory effect of NaCl (Table 19).

PROBLEM 5. What is the role of toxin internalization in the action of CTX?

See PROBLEM 4.

PROBLEM 6. What are the similarities and differences between CTXs and the presynaptically-acting snake venom PLA_2s?

Effects of neostigmine and nonradiolabeled choline on ^{14}C-choline and ^{14}C-acetylcholine release

Neostigmine at either 4 or 10 μM concentrations had no apparent effect on the release of either ^{14}C-choline or ^{14}C-acetylcholine, except for a possible slight decrease in choline release (Table 20). Therefore, the treatment with neostigmine was ignored and the data were grouped to compare the presence of choline on ^{14}C-choline and ^{14}C-acetylcholine release. The presence of unlabeled choline appeared to affect the action of β-Butx (Table 20). While the presence of unlabeled choline had very little effect on ^{14}C-choline release, ^{14}C-acetylcholine release was considerably increase in the absence or presence of β-Butx. The effect of unlabeled choline on ^{14}C-acetylcholine release seemed more dramatic in the presence of β-Butx (Table 20).

Time course of ^{14}C-choline and ^{14}C-acetylcholine release

In the rat synaptosomes, β-Butx and A23187 treatment differ from control release in that an early (2 min) stimulated release of ACh and choline is observed (Table 21). After 5 min a depression of *cumulative* release is

observed. In *mouse* synaptosomes (Table 22), the effects on depression of ACh release of the same concentration of β-Butx are less dramatic than in the rat preparations (Table 21). The effects on choline release in the mouse synaptosomes (Table 22) may be opposite to those in the rat synaptosomes (Table 21). These studies also have to be repeated. Oddly, the mouse control preparation, unlike the rat preparation, may stabilize and no longer exhibit net ACh or Ch release after 10 min (Table 22). This may affect the interpretation of the results.

Effects of a lipase inhibitor [p-bromophenacyl bromide (p-BPB)] on ^{14}C-choline and ^{14}C-acetylcholine release

The consequence of inhibition of PLA_2 activity during synaptosome isolation by p-BPB on ACh and Ch release was examined in purified synaptosomes (Table 23). There appeared to be no dramatic advantage in using this agent. However, the amount of ACh and Ch released was much higher than in the absence of p-BPB, suggesting that either the loading was improved or the leakage is enhanced by p-BPB. We have not verified that PLA_2 activity was significantly inhibited.

Time course of β-Butx- and A23187-induced ^{14}C-choline and ^{14}C-acetylcholine release from mouse synaptosomes

β-Butx (0.5 μM) and A23187 (20 μM) elicited time-dependent increases in ACh release relative to control preparations (Table 24). Both agents had very little effect on Ch release. Notice that these synaptosomes were prepared in p-BPB (100 μM)-containing medium.

Effects of Loading Conditions on ACh Release

Loading conditions dramatically influence the viability of the synaptosomes and the subsequent release of ACh and Ch (Table 25). While we intend to examine other loading conditions in the future, the use of 37°C, 20 μM ^{14}C-choline and a short loading period (30 min) appear to yield the best results.

Choline Uptake

Since we were experiencing considerable difficulties with the acetylcholine and choline release assays, we decided to examine the effects of low concentrations on toxin on choline uptake. At least for the two toxins tested (β-bungarotoxin and *Naja naja atra* PLA_2), the LD_{50} potencies correlated with the potencies in antagonizing choline uptake, based on rather preliminary findings (Table 26). This finding supports earlier studies suggesting inhibition of choline uptake may account for inhibition of acetylcholine release (Fletcher and Middlebrook, 1986).

Technical difficulties

We have experienced considerable variability in the effects of the toxins, high (40 mM) K^+ and A23187 on ACh and Ch release from the purified synaptosomal fractions. At times these agents have very marked effects on ACh release and at other times they have no effect. We have also noticed that the bands on the Percoll gradients are not uniform from centrifuge tube to centrifuge tube, despite similar protein loading from the same homogenate. Therefore, we tested the four major bands, as regards ACh and Ch release to determine if our problems might be related to variations in composition of

Band 3 - our normal synaptosome preparation (Table 27). It is clear from these results that only Band 3 yields results consistent with the recognized ACh release process. Therefore, our previous experience with inconsistent results could be due to the potential instability and possible overloading of the Percoll gradients.

Discussion and Conclusions:

Effects of snake venom CTXs on lipid metabolism in human red blood cells

Indirect approaches have suggested that the *Naja naja atra* and *Naja naja kaouthia* CTXs both possess a Ca^{2+}-dependent hemolytic action that may be attributed to venom PLA_2 contamination in the CTX fractions (Jiang et al., 1989). To more directly address this possibility, we have analyzed the free fatty acids produced by the CTX fractions in erythrocytes using gas chromatography. The fatty acid generating activity of the CTX fractions was completely eliminated under conditions (p-BPB-treated toxin) decreasing only the PLA_2 activity contaminating the CTX fractions. Under this condition much of the hemolytic activity of the *Naja naja kaouthia* CTX, but not the *Naja naja atra* CTX, remained. These studies support the hypothesis that there is a hemolytic component of at least one of the CTXs *not* dependent on PLA_2 activity, but that is enhanced by PLA_2 contamination.

Effects of snake venom CTXs and bee venom melittin on lipid metabolism in cell lines from airway epithelia and primary cultures from skeletal muscle

We found that in airway epithelial cells, 75% of the free fatty acid production formerly attributed to activation of PLA_2 by commercial melittin was due to contamination of the melittin fraction with bee venom PLA_2 protein (Fletcher et al., 1990d; and current Annual Report). In addition to producing free fatty acids, melittin and CTX elevate levels of diglycerides in airway epithelial cells (melittin) and skeletal muscle (melittin, CTX). The elevation of free fatty acids and diglycerides is even observed with synthetic melittin, ruling out contamination by the venom PLA_2 as the source.

The effects of CTX on normal muscle cannot be attributed to activation of tissue phospholipase A_2 activity, as suggested by other investigators (Mollay et al., 1976), for the following reasons: (1) the decrease in phospholipid as a percent of the total lipids observed with native toxin is antagonized by pretreating the toxin with p-BPB (Table 11); (2) even though the venom contaminant phospholipase A_2 activity in the toxin fraction is abolished by p-BPB treatment, the levels of free fatty acid and diglyceride formation are unaffected, suggesting that the bulk of the free fatty acids are derived from a nonphospholipid, nontriglyceride source; (3) the lack of specific phospholipid hydrolysis, as evidenced by no change in the % distribution of phospholipids. There is an asymmetry of phospholipid distribution in the membrane based on the preferred hydrolysis of phosphatidylcholine (PC) by *Naja naja atra* phospholipase A_2 contamination in the *Naja naja atra* CTX fraction (Table 10) and bee venom phospholipase A_2 contamination in the melittin fraction (Fletcher et al., 1990d and Table 13). This asymmetry should have led to preferential hydrolysis of the inner phospholipids (PI, PS, PE) by a tissue phospholipase A_2; (4) there is no evidence of lysophospholipid, although this is usually only observed at high levels of hydrolysis due to preferential radiolabeling of the unsaturated fatty acids at the #2 position of the

phospholipids. We speculate that *de novo* synthesis is the major source of free fatty acids in normal human skeletal muscle. Apparently triglyceride breakdown is not involved in the process of fatty acid production in normal skeletal muscle. In the case of skeletal muscle from malignant hyperthermia susceptible patients, triglyceride breakdown may also contribute to, or may simply parallel, free fatty acid and diglyceride synthesis.

Cardiotoxin and melittin interactions with snake and bee venom PLA_2 in artificial membranes

CTX and melittin share similar properties, including hemolysis of red blood cells and acting synergistically with PLA_2. Both toxins have been used as probes to activate tissue PLA_2 activity. The present study examined the interaction action between either CTX or melittin and snake or bee venom PLA_2. The maximum extent of hydrolysis of the substrate, not enzyme activity *per se* was studied on multilamellar and unilamellar vesicles to test if melittin or CTX increases the activity of the PLA_2 enzyme by simply increasing the substrate availability. Mixed micelles, which are readily hydrolyzed by both PLA_2s, were used to test if factors other than allowing bilayer penetration play a role in the interaction between PLA_2 and the CTXs and melittin. To avoid the confounding results obtained with trace PLA_2 contamination, melittin fractions were treated with p-BPB to inhibit PLA_2 activity. This treatment did not affect the hemolytic activity of melittin, but greatly reduced PLA_2 activity in the melittin fraction and, therefore, allowed the use of Ca^{2+} in the bathing medium.

Bee venom PLA_2 is incapable of penetrating either natural (Zwaal et al., 1975; Fletcher et al., 1987) or artificial (Sundler et al., 1978; Wilschut et al., 1979) bilayers. The predicted order of extent of substrate hydrolysis by this enzyme based on this simple fact is mixed micelle > unilamellar vesicles > multilamellar vesicles. The stimulation of PLA_2 activity by melittin would be predicted to be related inversely to the amount of substrate available to the enzyme. The multilamellar vesicles have the least substrate available and are the least readily hydrolyzed by the PLA_2 enzyme in the absence of melittin. If melittin simply formed a complex with phospholipids that makes them more susceptible to attack, then hydrolysis of mixed micelles would also be ennanced by the toxin. However, we observed no enhancement by melittin of the rate of PLA_2 catalyzed hydrolysis of either the egg yolk PC, or arachidonic acid-containing molecular species of PC.

While the formation of unilamellar vesicles from multilamellar vesicles (Dufourcq et al., 1986) might at least partially explain the activation of PLA_2 activity by melittin, the subsequent action of melittin on the bilayer is less clear. The effects of melittin on artificial substrates are complex and extrapolation of these results to biological substrates is difficult. PC bilayers can be converted to micelles (Dufourcq et al., 1986), whereas, cardiolipin (Batenburg et al., 1987a) and other negatively charged phospholipid (Batenburg et al., 1987b) vesicles can be converted to the H_{II} conformation. In contrast, melittin inhibits the natural H_{II} phase formation typical of phosphatidylethanolamine and stabilizes bilayer formation (Batenburg et al., 1988). Dipalmitoyl PC and distearoyl PC mixtures do not exhibit phase separation on addition of melittin; however, addition of dipalmitoylphophatidylglycerol to either of these phospholipids does result in phase separation (Lafleur et al., 1989). Formation of micelles (Dufourcq et

al., 1986) at small sites on the membrane does not fully explain the complete hydrolysis of the substrates observed in the present study. However, it is possible that hydrolysis of a region around a discoidal partical could allow penetration of the PLA_2 enzyme into the interior of the bilayer and then allow complete hydrolysis of the interior phospholipids.

The present study demonstrates that melittin does not at all enhance the action of bee venom PLA_2 on mixed micelle substrates in the absence of NaCl, but does increase bee venom PLA_2 activity on mixed micelles in the presence of NaCl. These findings bring into question the use of melittin as an activator of endogenous PLA_2 activity, due to the extreme lack of specificity of this action, the high levels of phospholipid hydrolysis due to trace contamination with bee venom PLA_2 and the apparent disruption of the membrane bilayer by the toxin.

Our studies suggest that melittin and CTX act by similar, but not identical mechanisms, based on similarities and differences in their interactions with PLA_2 and differences in effects on lipid metabolism in cell cultures. Certainly bee venom and snake venom PLA_2s differ markedly in their interactions with CTXs and melittin. The *Naja naja kaouthia* CTX and melittin do possess activities not dependent on venom PLA_2 contamination in the CTX fraction. These toxins both elevate free fatty acids and diglycerides. At this time we believe these elevated neutral lipids to be related to activation of *de novo* synthesis of fatty acids by the CTXs.

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TABLE 1. Distribution of radiolabel in each cell line.

BEAS-2

	Phospholipids											
Toxin	LPC	SM	PC	PI	PS	PE	PA	CL	PL	DG	FFA	TG
	(% of Phospholipid Labeled)								(% Total Label)			
Control	1.4	5.0	67	11	3.6	12	0.0	0.0	90	3.1	1.3	5.4
BV PLA_2 2 nM	9.3	5.8	54	14	3.7	12	0.0	0.8	66	3.1	18	12
Melittin 2 µM	21	9.7	32	19	3.4	8.8	2.9	3.6	36	4.2	51	8.7
p-BPB Mel 2 µM	1.5	3.2	53	11	2.8	21	1.6	5.1	78	4.3	10	7.2
p-BPB Mel + PLA_2	16	7.0	23	21	3.2	10	4.7	15	42	4.0	46	7.6

CFNP1

	Phospholipids											
Toxin	LPC	SM	PC	PI	PS	PE	PA	CL	PL	DG	FFA	TG
	(% of Phospholipid Labeled)								(% Total Label)			
Control	0.7	2.2	45	9.0	3.5	30	2.0	8.4	88	3.6	3.4	5.0
BV PLA_2 2 nM	4.9	3.3	38	12	3.7	23	3.1	12	65	4.1	24	7.3
Melittin 2 µM	11	6.3	24	18	3.3	12	5.8	19	44	5.3	46	3.3
p-BPB Mel 2 µM	2.0	4.0	59	14	2.9	14	0.8	4.0	75	4.4	15	4.8
p-BPB Mel + PLA_2	20	9.5	29	23	2.8	9.1	2.6	4.1	36	4.6	53	5.5

NNM3

	Phospholipids											
Toxin	LPC	SM	PC	PI	PS	PE	PA	CL	PL	DG	FFA	TG
	(% of Phospholipid Labeled)								(% Total Label)			
Control	0.5	1.3	63	7.4	3.0	19	0.0	5.4	88	2.1	0.8	8.7
BV PLA_2 2 nM	1.9	2.0	56	9.6	3.1	17	1.1	9.2	77	2.2	7.0	14
Melittin 2 µM	5.6	2.5	45	10	2.4	16	2.0	17	51	3.3	25	21
p-BPB Mel 2 µM	0.5	0.0	53	6.5	2.7	20	1.5	15	81	3.0	4.4	11
p-BPB Mel + PLA_2	3.9	1.8	42	9.8	2.9	16	2.6	21	53	3.4	23	20

TABLE 2. Distribution of ^{14}C-linoleic acid in lipid extracts (total counts), total phospholipids (PL) and into specific neutral lipid fractions of immortalized airway epithelial cells after incubation in the absence or presence of toxin.

Treatment	PL	DG	FFA	TG	Total Counts
	[% Total Label (mean±SEM)]				[CPM (mean±SEM)]
Control	$89\pm1^{a,b,c}$	2.9 ± 0.4	$1.8\pm0.8^{h,i}$	6.4 ± 1.2	139,000± 7,000
BV PLA_2 2 nM	$69\pm4^{a,d,e}$	3.1 ± 0.5	$16\pm5^{j,k}$	11 ± 2	134,000±11,000
Melittin 2 µM	$44\pm4^{b,d,f}$	4.3 ± 0.6	$41\pm8^{h,j,l}$	11 ± 5	137,000±11,000
p-BPB Mel 2 µM	$78\pm2^{f,g}$	3.9 ± 0.5	$9.8\pm3.1^{l,m}$	7.7 ± 1.8	134,000± 8,000
p-BPB Mel + PLA_2	$44\pm5^{c,e,g}$	4.0 ± 0.3	$41\pm9^{i,k,m}$	11 ± 5	126,000± 9,000

Cells were prelabeled with ^{14}C-linoleic acid for 22 hrs. The cells were then incubated in the absence or presence of toxin for 1 hr at 37°C, lipids extracted, separated by 1-D TLC (Fig. 1A) and radioactivity in each peak integrated.

Differences were tested for each lipid by a one-way ANOVA and Newman-Keuls test. Significantly different values indicated by identical superscripts by $P<.05$ (j,k,l,m) or $P<.01$ (a,b,c,d,e,f,g,h,i).

Abbreviations: see Fig. 2.

TABLE 3. Distribution of ^{14}C-linoleic acid in specific phospholipids of immortalized airway epithelial cells after incubation in the absence or presence of toxin.

Treatment	Phospholipids							
	LPC	SM	PC	PI	PS	PE	PA	CL
	[% of Phospholipid Labeled (mean±SEM)]							
Control	0.9±0.3	2.8±1.1	58±7[a,b]	9±1	3.4±0.2	20±5	0.6±0.6	4.6±2.5
BV PLA_2 2 nM	5.4±2.1	3.7±1.1	49±6	12±1	3.5±0.2	17±3	1.4±0.9	7.3±3.4
Melittin 2 µM	12±5	6.2±2.1	34±6[a,c]	16±3	3.0±0.3	12±2	3.6±1.1	13±5
p-BPB Mel 2 µM	1.3±0.4	2.4±1.2	55±2[c]	11±2	2.8±0.1	18±2	1.3±0.3	8.0±3.5
p-BPB Mel + PLA_2	13±5	6.1±2.3	31±6[b]	18±4	3.0±0.1	12±2	3.3±0.7	13±5

Cells were prelabeled with ^{14}C-linoleic acid for 22 hrs. The cells were then incubated in the absence or presence of toxin for 1 hr at 37°C, lipids extracted, separated by 1-D TLC (Fig. 1B) and radioactivity in each peak integrated.

Differences were tested for each phospholipid by a one-way ANOVA and Newman-Keuls test. Significantly different values indicated by identical superscripts by $P<.05$.

Abbreviations: see Fig. 2.

TABLE 4. Uptake of different radiolabeled fatty acids into human primary muscle cell culture. Each set of values reflects a different culture dish (i.e., n=1 for each condition). These primary cultures were obtained from a different investigators (T. Heiman-Patterson) than those in Table 1 (Q.H. Gong).

Toxin	Phospholipids								Neutrals					Total
	LPC	SM	PC	PI	PS	PE	PA	CL	PL	DG	FFA	TG	CHE	Counts
	(% of Phospholipid Labeled)								(% Total Label)					
18:0														
24 hr	0.0	0.0	45	16	8.0	13	6.1	13	84	0.0	0.0	16	0.0	2,915
48 hr	0.0	0.0	45	14	11	19	4.4	6.0	94	0.0	0.0	5.7	0.0	5,256
72 hr	0.0	0.0	50	8.8	3.4	12	11	15	88	0.0	3.8	8.1	0.0	6,133
18:1														
24 hr	0.0	0.0	57	--4.9--		13	--25--		84	0.0	0.0	16	0.0	8,085
48 hr	0.0	0.0	61	2.7	4.2	12	1.4	18	86	0.0	0.0	12	1.9	9,134
72 hr	1.6	0.0	63	2.1	4.0	7.5	--21--		90	0.0	0.0	10	0.0	6,557
18:2														
24 hr	0.0	0.0	71	4.0	1.9	8.0	4.0	12	69	0.0	0.0	28	2.6	21,290
48 hr	1.7	0.0	66	2.9	3.4	8.0	4.1	14	71	0.0	0.0	28	1.3	18,608*
72 hr	0.0	0.0	66	2.7	1.3	7.2	2.9	20	76	0.0	0.0	21	2.7	21,393
18:3														
24 hr	0.0	0.0	65	2.0	0.9	8.4	6.9	17	46	0.0	0.0	50	3.4	20,199
48 hr	0.0	0.0	61	2.7	2.8	13	6.6	14	79	0.0	0.0	21	0.0	11,581*
72 hr	0.0	0.0	69	2.3	0.0	4.3	4.2	20	66	0.0	0.0	28	5.1	5,684

Cells were prelabeled with fatty acids for 24-72 hrs. The lipids were then extracted, separated by 1-D TLC and radioactivity in each peak integrated.

--XX-- indicates that two peaks were not resolved and were integrated as a single peak.

TABLE 5. Distribution of radiolabel in each human primary muscle cell culture. Cultures were exposed to *Naja naja kaouthia* CTX (3 μM) for 2 hr (37°C). Each set of values reflects a different culture dish (i.e., n=2 for each condition).

	Phospholipids												
Toxin	LPC	SM	PC	PI	PS	PE	PA	CL	PL	DG	FFA	TG	CHE
	(% of Phospholipid Labeled)								(% Total Label)				
Control	0.0	2.1	66	3.2	3.2	7.9	6.0	12	77	0.9	0.0	19	3.3
	0.0	2.4	67	3.3	4.3	6.4	5.1	11	79	0.8	0.0	18	3.0
N.n. kaouthia CTX	0.0	2.7	69	3.2	4.2	10	4.3	6.7	75	1.8	1.8	19	2.5
	0.0	2.3	70	3.2	4.1	9.2	4.9	6.6	74	2.1	1.8	20	2.6
p-BPB CTX	0.0	2.7	68	3.7	5.3	10	4.6	5.5	76	1.8	1.7	19	2.1
	0.0	2.1	67	2.8	4.3	8.5	6.0	9.2	72	2.1	1.8	22	1.4

Cells were prelabeled with ^{14}C-linoleic acid for 22 hrs. The cells were then incubated in the absence or presence of toxin for 2 hr at 37°C, lipids extracted, separated by 1-D TLC and radioactivity in each peak integrated.

TABLE 6. Distribution of radiolabel in each human primary muscle cell culture. Cultures were exposed to p-bromophenacyl bromide-treated *Naja naja kaouthia* CTX (3 μM) for 5 to 60 min (37°C). Each set of values reflects a different culture dish (i.e., n=2 for each condition). Note: This culture was used at approximately 1 wk after plating. In contrast, the culture in the previous quarterly report was used at about 2 wks after plating.

	Phospholipids												
Toxin	LPC	SM	PC	PI	PS	PE	PA	CL	PL	DG	FFA	TG	CHE
	(% of Phospholipid Labeled)								(% Total Label)				
Control	0.0	1.9	65	2.8	4.5	19	0.6	6.4	68	1.2	0.4	29	1.4
	0.0	2.4	67	1.5	3.9	18	0.5	6.3	72	1.3	0.6	25	1.7
N.n. kaouthia CTX	-	-	-	-	-	-	-	-	73	1.3	0.7	23	1.3
(5 min)	-	-	-	-	-	-	-	-	-	-	-	-	-
N.n. kaouthia CTX	0.0	2.0	67	1.7	4.0	18	0.0	6.8	78	1.1	1.2	19	0.8
(10 min)	0.0	2.5	65	2.1	4.6	18	0.0	6.9	74	1.4	0.8	23	1.1
N.n. kaouthia CTX	0.0	1.8	65	2.5	4.9	19	0.6	5.6	70	1.3	1.2	27	1.1
(30 min)	0.0	2.2	64	1.9	4.7	19	0.7	7.3	69	1.4	1.0	28	0.3
N.n. kaouthia CTX	0.0	2.5	68	1.9	3.8	18	0.0	6.5	73	2.1	1.6	23	0.2
(60 min)	0.0	2.1	65	2.7	4.3	19	0.0	7.0	73	2.3	2.3	22	0.3

Cells were prelabeled with ^{14}C-linoleic acid for 72 hrs. The cells were then incubated in the absence or presence of toxin for 1 hr at 37°C, lipids extracted, separated by 1-D TLC and radioactivity in each peak integrated.

- indicates sample was lost in processing.

TABLE 7. Distribution of radiolabel in each human primary muscle cell culture. Cultures were exposed to native *Naja naja kaouthia* CTX for 60 min or p-bromophenacyl bromide-treated CTX (3 μM) for 5 to 60 min (37°C). Each set of values reflects a different culture dish (i.e., n=2 for each condition). Note: This culture was used at approximately 2 wks after plating. In contrast, the culture in Table 6 was used at about 1 wk after plating.

	Phospholipids								Neutral Lipids				
Toxin	LPC	SM	PC	PI	PS	PE	PA	CL	PL	DG	FFA	TG	CHE
	(% of Phospholipid Labeled)								(% Total Label)				
Control	0.5	2.0	61	2.9	5.0	14	1.0	14	81	0.7	0.0	18	1.1
	0.0	2.0	65	1.5	3.6	16	0.0	13	82	0.0	0.8	15	2.3
N.n. kaouthia CTX	0.0	2.1	64	2.2	3.8	16	1.5	10	80	0.6	1.0	17	1.7
(5 min)	-	-	-	-	-	-	-	-	80	0.4	0.4	19	0.0
N.n. kaouthia CTX	0.0	1.6	62	3.3	4.8	16	1.1	11	75	0.5	0.7	22	1.9
(30 min)	0.0	1.7	61	3.4	4.9	17	0.9	12	78	0.0	0.6	19	2.3
N.n. kaouthia CTX	0.0	2.1	63	1.9	3.6	17	0.0	12	82	0.6	1.0	13	2.5
(60 min)	0.0	2.3	61	2.0	5.0	17	1.2	12	83	0.7	1.0	13	2.5
N.n. kaouthia CTX	0.0	2.2	60	2.9	5.0	17	0.7	12	77	0.9	1.4	18	2.6
(60 min)-native	0.0	2.1	62	1.8	4.1	15	1.2	14	77	0.7	1.5	18	3.3

Cells were prelabeled with ^{14}C-linoleic acid for 72 hrs. The cells were then incubated in the absence or presence of toxin for 1 hr at 37°C, lipids extracted, separated by 1-D TLC and radioactivity in each peak integrated.

TABLE 8. Effects of *Naja naja kaouthia* CTX on lipid metabolism in human primary skeletal muscle cell cultures. Examples of two types of responses to the toxin are indicated based on cultures from two different patients, one of which has a skeletal muscle disorder that results in altered lipid metabolism. Confluent cell cultures were radiolabeled to equilibrium with linoleic acid (3 days). Cultures (35 mm dishes) were then incubated at 37°C for 2 hrs with or without (control) native or p-bromophenacyl bromide (p-BPB) treated *Naja naja kaouthia* CTX (10 μM).

	Neutral Lipids				
	PL	DG	FFA	TG	CHE
	(% Distribution; mean ± SEM; n=3)				
Control Patient					
No CTX	82±1	0.2±0.2	0.0±0.0	16±1	2.1±0.2
p-BPB CTX	67±4[b]	3.2±0.8[b]	9.3±0.9[d]	20±3	0.9±0.1[c]
native CTX	70±2[a]	3.9±0.3[c]	8.0±1.0[d]	18±1	0.2±0.2[d]
MH Patient					
No CTX	69±1	0.0±0.0	0.0±0.0	28±1	2.8±0.2
p-BPB CTX	66±1	3.7±0.3[e]	6.0±0.4[e]	22±2[a]	2.3±0.1
native CTX	71±1[a,b]	4.1±0.0[e]	6.9±0.4[e]	17±1[c,a]	0.2±0.1[e]

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	Phospholipids							
	LPC	SM	PC	PI	PS	PE	PA	CL
	(% of Phospholipid Labeled)							
Control Patient								
No CTX	0.0±0.0	2.3±0.1	60±1	4.4±0.1	5.2±0.1	20±1	1.2±0.1	7.3±0.1
p-BPB CTX	0.0±0.0	2.0±0.1	62±2	2.4±0.5[a]	3.1±0.4[a]	20±1	1.1±0.6	9.2±1.3
native CTX	0.0±0.0	2.2±0.1	63±1	2.6±0.4	3.4±0.5	19±0	1.4±0.1	7.9±0.8
MH Patient								
No CTX	0.0±0.0	2.5±0.3	63±0	3.3±0.8	4.0±0.4	16±1	1.7±0.2	9.2±0.4
p-BPB CTX	0.0±0.0	2.7±0.2	60±1	5.1±0.2	5.0±0.4	14±0	2.9±0.1[c]	10±2
native CTX	0.0±0.0	2.7±0.3	63±1	4.5±0.5	5.3±0.2	14±1	1.8±0.2*[c]	9.3±1.0

Abbreviations: PL, phospholipid; DG, diacylglyceride; FFA, free fatty acid; TG, triacylglyceride; CHE, cholesterol esters; Ori, origin; LPC, lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PA, phosphatidic acid; CL, cardiolipin; NL, neutral lipid.

ANOVA/Scheffe
[a]=.05; [b]=.02; [c]=.01; [d]=.001; [e]=.0001 different from control.
*=> difference between CTXs.

TABLE 9. Effects of *Naja naja kaouthia* CTX on lipid metabolism in control human primary skeletal muscle cell cultures from a patient with high level of radiolabeling of the triglycerides. Confluent cell cultures were radiolabeled to equilibrium with linoleic acid (3 days). Cultures (35 mm dishes) were then incubated at 37°C for 2 hrs with or without (control) native or p-bromophenacyl bromide (p-BPB) treated *Naja naja kaouthia* CTX (10 µM).

	Neutral Lipids				
	PL	DG	FFA	TG	CHE
	(% Distribution; mean of triplicate determinations)				
No toxin	49	0.0	0.0	48	3.5
p-BPB toxin	46	1.6	2.2	47	3.3
native toxin	48	2.0	3.2	45	1.7

Note: Implies that the effects on TGs are not related to amount of TG present.

	Phospholipids							
	LPC	SM	PC	PI	PS	PE	PA	CL
	(% of Phospholipid Labeled; mean of triplicate determinations)							
	LPC	SM	PC	PI	PS	PE	PA	CL
No toxin	0.0	1.2	75	6.7	5.1	8.7	1.5	1.9
p-BPB toxin	0.0	1.2	67	6.1	5.4	11	2.9	6.7
native toxin	0.6	1.0	63	5.0	5.6	12	3.1	10

TABLE 10. Effects of *Naja naja atra* CTX on lipid metabolism in human primary skeletal muscle cell cultures from a patient with malignant hyperthermia. Confluent cell cultures were radiolabeled to equilibrium with linoleic acid (3 days). Cultures (35 mm dishes) were then incubated at 37°C for 2 hrs with or without (control) native or p-bromophenacyl bromide (p-BPB) treated *Naja naja atra* CTX (10 μM).

	Neutral Lipids				
	PL	DG	FFA	TG	CHE
	(% Distribution; mean ± SD; n=3)				
No CTX	70±3	0.0±0.0	0.0±0.0	28±3	1.7±0.3
p-BPB CTX	50±2	4.4±0.7	14±1	30±4	1.8±0.8
native CTX	17±1	4.0±0.3	55±7	22±6	1.8±1.6

	Phospholipids							
	LPC	SM	PC	PI	PS	PE	PA	CL
	(% of Phospholipid Labeled)							
No CTX	0.0±0.0	2.8±0.7	68±6	3.5±0.3	4.5±0.7	12±3	2.2±0.2	7.2±2.4
p-BPB CTX	0.0±0.0	3.1±0.8	61±2	4.4±0.4	5.7±0.2	13±1	2.2±2.0	10.6±0.6
native CTX	13	10	32	8.6	6.6	11	0.0	18

TABLE 11. Effects of *Naja naja kaouthia* CTX on lipid metabolism in human primary skeletal muscle cell cultures. Examples of two types of response to the toxin are indicated based on cultures from two different patient populations, one of which has a skeletal muscle disorder that results in altered lipid metabolism. Confluent cell cultures were radiolabeled to equilibrium with linoleic acid (3 days). Cultures (35 mm dishes) were then incubated at 37°C for 2 hrs with or without (control) native or p-bromophenacyl bromide (p-BPB) treated *Naja naja kaouthia* CTX (10 μM).

		Neutral Lipids				
	n	PL	DG	FFA	TG	CHE
		(% Distribution; mean ± SEM)				
Control						
No toxin	4	80±2	0.05±0.05	0.18±0.12	18±2	2.1±0.6
p-BPB toxin	4	74±2	3.6±0.3[b]	6.1±1.1[b]	16±2	1.1±0.6
native toxin	4	72±1[a]	3.6±0.3[b]	6.6±0.6[b]	17±1	0.7±0.4

[a]Different from no toxin (P<.05) by 1-way ANOVA and Scheffe test.
[b]Different from no toxin (P<.001) by 1-way ANOVA and Scheffe test.

MH susceptible (Note: since n=2, no statistical tests done)						
No toxin	2	76±7	0.00±0.00	0.00±0.00	22±7	2.4±0.4
p-BPB toxin	2	72±6	3.5±0.3	6.0±0.0	18±5	1.8±0.6
native toxin	2	77±6	4.1±0.1	6.2±0.8	12±5	0.3±0.1

	Phospholipids							
n=4	LPC	SM	PC	PI	PS	PE	PA	CL
	(% of Phospholipid Labeled; mean ± SEM)							
Control								
No toxin	0±0	2.6±0.1	64±2	4.0±0.2	4.6±0.4	15±2		11±1
p-BPB toxin	0±0	2.8±0.4	62±2	3.3±0.4	4.3±0.5	15±2		13±1
native toxin	0±0	2.6±0.4	63±2	3.8±0.7	4.6±0.4	15±2		11±2

Note: "n" refers to number of patients. The value for each patient was derived from the mean of three determinations.

TABLE 12. Effects of *Naja naja kaouthia* CTX on lipid metabolism in equine primary skeletal muscle cell cultures. Confluent cell cultures were radiolabeled to equilibrium with linoleic acid (3 days). Cultures (35 mm dishes) were then incubated at 37°C for 2 hrs with or without (control) native or p-bromophenacyl bromide (p-BPB) treated *Naja naja kaouthia* CTX (10 µM).

	Neutral Lipids				
	PL	DG	FFA	TG	CHE
	(% Distribution; mean of triplicate determinations)				
Normal Horses					
No toxin					
Horse # 1	82	0.0	0.0	17	0.8
Horse # 2	92	0.0	0.0	7.8	0.6
p-BPB toxin					
Horse # 1	76	2.6	2.6	19	0.3
Horse # 2	86	2.6	4.3	6.7	0.5
native toxin					
Horse # 1	75	2.7	4.2	18	0.6
Horse # 2	-	-	-	-	-
Hyperkalemic Periodic Paralysis Horse					
	PL	DG	FFA	TG	CHE
	(% Distribution; individual values for duplicate determinations)				
No toxin	66;70	0.0;0.0	0.0;0.0	34;28	0.0;1.3
p-BPB toxin	58;70	0.0;0.0	3.8;2.1	36;27	1.8;1.0
native toxin	68;68	0.0;0.0	1.9;2.9	29;28	1.1;0.0

	Phospholipids							
Normal Horse	LPC	SM	PC	PI	PS	PE	PA	CL
	(% of Phospholipid Labeled; mean of triplicate determinations)							
No toxin								
Horse # 1	0.0	0.8	53	8.9	6.0	20		12
Horse # 2	0.0	0.7	59	6.7	5.7	17		11
p-BPB toxin								
Horse # 1	0.0	0.0	56	9.0	5.5	19		10
Horse # 2	0.0	0.6	61	6.2	5.8	15		11
native toxin								
Horse # 1	0.0	0.0	54	8.9	5.6	21		11
Horse # 2	0.0	-	-	-	-	-		-
Hyperkalemic Periodic Paralysis Horse	LPC	SM	PC	PI	PS	PE	PA	CL
	(% of Phospholipid Labeled; individual values for duplicate determinations)							
No toxin	0;0	2.5;2.3	50;50	6.4;7.5	5.5;6.3	26;24	6.5;6.0	2.9;4.2
p-BPB toxin	0;0	2.3;1.3	57;52	4.2;4.2	4.6;6.2	20;24	7.1;8.3	4.8;3.9
native toxin	0;0	1.6;1.7	49;51	6.8;6.2	6.4;4.7	22;23	8.7;8.9	5.4;4.3

TABLE 13. Effects of bee venom melittin on lipid metabolism in human primary skeletal muscle cell cultures. Examples of two types of responses to the toxin are indicated based on cultures from two different patients, one of which has a skeletal muscle disorder that results in altered lipid metabolism. Confluent cell cultures were radiolabeled to equilibrium with linoleic acid (3 days). Cultures (35 mm dishes) were then incubated at 37°C for 2 hrs with or without (control) synthetic or p-bromophenacyl bromide (p-BPB) treated Sigma melittin.

	Neutral Lipids				
	PL	DG	FFA	TG	CHE
	(% Distribution; mean of duplicate/triplicate)				
Melittin (2 μM)					
Control					
No toxin	73	0	0	26	1.2
p-BPB toxin (Sigma)	73	4.3	7.3	15	0.4
synthetic toxin	73	4.0	4.9	17	1.4
MH Susceptible					
No toxin	57	0	0	43	0.4
p-BPB toxin (Sigma)	61	2.8	10	26	0.4
synthetic toxin	57	3.1	8.3	30	1.0
Melittin (10 μM)					
Control					
No toxin	73	0	0	26	1.2
p-BPB toxin (Sigma)	61	8.5	24	6.8	0.0
synthetic toxin	75	7.0	9.5	7.5	0.6

	Phospholipids							
	LPC	SM	PC	PI	PS	PE	PA	CL
	(% of Phospholipid Labeled)							
Melittin (2 μM)								
Control								
No toxin	0.6	2.1	61	5.3	4.9	13	10	
p-BPB toxin	0.3	2.3	57	5.4	5.4	17	13	
synth. toxin	0.0	2.8	58	5.4	5.2	16	13	
MH Susceptible								
No toxin	0.0	1.5	68	6.0	5.7	11	7.5	
p-BPB toxin	0.0	1.9	60	6.3	7.3	16	9.1	
synth. toxin	0.0	1.7	63	5.7	6.8	14	9.1	
Melittin (10 μM)								
Control								
No toxin	0.6	2.1	61	5.3	4.9	16	10	
p-BPB toxin	0.0	3.2	53	7.8	7.0	17	12	
synth. toxin	0.0	2.6	59	6.1	5.1	18	9.6	

TABLE 14. Free fatty acid release from aged red blood cells incubated with various snake venom toxins in a Ca^{2+} or Sr^{2+} containing medium

		Free Fatty Acid							
Toxin	Conc.	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
Ca^{2+}	(μM)	(pmol/mg RBC protein; mean±SD)							
None	-	12± 3	144±15	10± 9	101±11	123± 17	30± 11	8± 0	14± 5
$BVPLA_2$	1	19± 2	433±49	50±18	161±75	1681± 74	1602± 56	25±10	3282±334
$SVPLA_2$	1	27± 5	515±47	40± 7	129±15	1940±198	1971±166	23± 3	3911±410
NnkCTX	30	16±11	146±47	3± 4	99±11	165± 19	99± 15	8± 2	222± 37
NnaCTX	30	20±16	168± 2	19±13	117±42	681± 44	623±108	13± 0	1430±123
NnkCTXB	30	9± 2	103±12	6± 2	80±10	88± 30	23± 10	7± 2	19± 19
NnaCTXB	30	10± 1	110±31	4± 1	86±10	74± 28	20± 10	7± 2	12± 6

TABLE 15. Effects of time to complete extraction and EGTA addition on PLA_2 activity results using ULVs and buffer containing Ca^{2+} 0 mM and NaCl 0 mM. Note that immediate complete extraction is required.

[PLA_2] and Time incubated	Incubations start at T=0, extracted at T=4 hrs[a]	Incubations start at T=0, EGTA to stop extracted at T=4 hrs[b]	Incubations extracted immediately[c]
		% Hydrolysis[d]	
10 nM			
1 hr	17.9	6.2	10.7
2 hr	32.9	8.6	11.1
3 hr	44.2	7.0	11.6
4 hr	20.9	16.0	10.7
100 nM			
1 hr	44.4	19.6	27.3
2 hr	41.3	19.2	32.0
3 hr	33.5	18.1	30.1
4 hr	28.5	24.0	31.9
1000 nM			
1 hr	86.7	28.0	45.8
2 hr	82.5	31.4	47.9
3 hr	74.9	30.5	52.3
4 hr	56.7	53.4	52.3

[a]The incubations were all started at the same time. Methanol was added after the designated incubation period to stop the reaction. All the incubations were extracted 4 hrs after they had begun.

[b]The incubations were all started at the same time. EGTA (10 mM) was added after the designated incubation period to stop the reaction. All the incubations were extracted 4 hrs after they had begun.

[c]The incubations were all started at the same time. All the incubations were extracted immediately after the indicated incubation period.

[d]Values are the average of duplicate incubations.

TABLE 16. Hydrolysis of ULVs by *Naja naja atra* PLA_2. Levels of hydrolysis are stable for 1 to 4 hrs at the concentrations below. Substrates #1 and #2 are identical, except that they were made on different days.

Concentration	% Hydrolysis Ca^{2+}(2 mM) NaCl(130 mM)	Ca^{2+}(0 mM) NaCl(0 mM)
Substrate #1		
10 nM	20	11
100 nM	31	20
1 µM	65	46
3 µM	79	-
Substrate #2		
3 µM	69	-
3 µM (bee venom)	85	-

TABLE 17. Dose-hydrolysis relationship for high concentrations of *Naja naja atra* PLA_2 using ULVs, Ca^{2+} 2 mM, NaCl 130 mM, 2 hr, 37°C.

Concentration	% Hydrolysis
1 µM	55.0
5 µM	64.4
10 µM	68.8

TABLE 18. Effects of BSA (0.5%) on hydrolysis of ULVs by *Naja naja atra* PLA_2.

PLA_2 Concentration	% Hydrolysis No BSA	BSA (0.5%)
1 nM	10	32
10 nM	13	34
100 nM	24	52
1 µM	60	65

TABLE 19. Effects of BSA, Ca^{2+} and NaCl on the interaction between snake venom PLA_2 (10 nM) and 1 and 10 μM concentrations of p-BPB-treated cardiotoxin (*Naja naja kaouthia*). PLA_2 was present under all conditions.

[CTX μM]	[BSA %]	[Ca^{2+} mM]	[NaCl mM]	% Hydrolysis
0	0	0	0	13
			130	12
		2	0	20
			130	18
1		0	0	32
			130	17
		2	0	36
			130	36
10		0	0	46
			130	13
		2	0	53
			130	52
0	0.5	0	0	28
			130	24
		2	0	45
			130	41
1		0	0	31
			130	25
		2	0	39
			130	42
10		0	0	23
			130	14
		2	0	68
			130	67

TABLE 20. Effects of neostigmine (4 or 10 μM) and nonradiolabeled choline (10 μM) on ^{14}C-choline and ^{14}C-acetylcholine release from *rat* brain synaptosomes. Following a 30 min ^{14}C-choline (10 μM) uptake period (37°C), highly purified synaptosomes were incubated for 5 min at 37°C with or without β-bungarotoxin (β-Butx; 1 μM) and radiolabeled choline and acetylcholine in the supernatant subsequently determined by radioimaging scanning of a TLC plate.

Concentration of Agent in μM		Control		β-Butx	
choline	neostigmine	Ach	Ch	Ach	Ch
		Counts		Counts	
10	0	841	503	896	431
0	0	640	301	417	302
10	4	733	320	827	280
0	4	502	123	594	375
10	10	770	430	829	430
0	10	465	324	479	372
		mean±SD		mean±SD	
10	0,4,10	781±55	418± 92	851±39	380±87
0	0,4,10	536±92	249±110	497±90	350±41

TABLE 21. Time course of ^{14}C-choline and ^{14}C-acetylcholine release from *rat* brain synaptosomes. Following a 30 min ^{14}C-choline (10 μM) uptake period (37°C), highly purified synaptosomes were incubated in the presence of choline (20 μM) for various times at 37°C with or without β-bungarotoxin (β-Butx; 1 μM) and radiolabeled choline and acetylcholine in the supernatant subsequently determined by radioimaging scanning of a TLC plate.

	Counts					
	Control		β-Butx		A23187 (20 μM)	
Time (min)	Ach	Ch	Ach	Ch	Ach	Ch
2	0	0	369	237	501	842
5	599	354	576	93	459	1497
10	902	525	652	302	860	1707
30	1738	1356	887	1002	809	3862

TABLE 22. Time course of ^{14}C-choline and ^{14}C-acetylcholine release from *mouse* brain synaptosomes. Following a 30 min ^{14}C-choline (10 μM) uptake period (37°C), highly purified synaptosomes were incubated in the presence of choline (20 μM) for various times at 37°C with or without β-bungarotoxin (β-Butx; 1 μM) and radiolabeled choline and acetylcholine in the supernatant subsequently determined by radioimaging scanning of a TLC plate.

	Counts			
	Control		β-Butx	
Time (min)	Ach	Ch	Ach	Ch
2	428	300	558	632
10	1094	776	725	641
30	1383	708	1175	1183

TABLE 23. Effects of isolation of mouse brain synaptosomes (Band 3) in the presence of p-BPB on the time course of ^{14}C-choline and ^{14}C-acetylcholine release. Following a 30 min ^{14}C-choline (10 μM) uptake period (37°C), fractions were incubated for various times in buffer containing choline (10 μM) and neostigmine (4 μM) at 37°C with or without high K^+ (40 mM) and radiolabeled choline and acetylcholine in the supernatant subsequently determined by radioimaging scanning of a TLC plate.

	Counts			
	Control		High K^+	
Time (min)	ACh	Ch	ACh	Ch
No p-BPB				
0	313	320	521	712
2	380	379	985	660
5	376	532	1162	721
10	582	834	936	239
30	501	938	843	100
p-BPB (100 μM)				
0	419	345	374	1773
2	545	414	1439	1218
5	638	524	1316	1692
10	733	1051	881	815
30	668	1241	1140	961

TABLE 24. Time course of ^{14}C-choline and ^{14}C-acetylcholine release from mouse brain synaptosomes (Band 3). Following a 30 min ^{14}C-choline (10 µM) uptake period (37°C), fractions were incubated for various times in buffer containing choline (10 µM) and neostigmine (4 µM) at 37°C with or without β-bungarotoxin (β-Butx; 0.5 µM) or A23187 (20 µM) and radiolabeled choline and acetylcholine in the supernatant subsequently determined by radioimaging scanning of a TLC plate.

	Counts					
	Control		β-Butx		A23187 (20 µM)	
Time (min)	ACh	Ch	ACh	Ch	ACh	Ch
0	550	477	486	507	568	464
2	600	472	789	808	374	369
5	471	-	1078	1165	946	318
10	488	597	1220	1700	2508	590
30	1239	2047	1615	1861	1760	1084

TABLE 25. Test of loading conditions on subsequent release of ACh and Ch. Synaptosomal fractions were loaded with either 10 or 20 µM ^{14}C-choline at 37°C (30 or 60 min) or 25°C (60 min) and then incubated for 5 min in buffer containing either low (2.7 mM) or high K^+. Preparations were centrifuged and the radioactivity in the supernatants determined.

Conc. (µM)	Uptake		Release	Release of Radioactivity (Counts)	
^{14}C-choline	Temp (°C)	Time (min)	Medium [K^+]	ACh	Ch
10	37	30	low	1764	623
				1258	473
20				978	470
				1271	449
10			high	2430	730
				1567	2535
20				2433	976
				3145	943
10	37	60	low	-	-
				-	-
20				231	414
				-	-
10			high	683	1189
				-	1482
20				212	464
				295	632
10	25	60	low	413	377
				594	353
20				1179	399
				1169	406
10			high	501	339
				565	628
20				908	719
				1265	532

- Radiochromatogram unintelligible.

TABLE 26. Effects of β-bungarotoxin and *Naja naja atra* PLA_2 on choline uptake in mouse brain synaptosomes. High affinity choline uptake in the presence of toxin was compared to that in the absence of toxin and the % of control calculated from the means of triplicate determinations. Values are presented as the mean of triplicate determinations or the mean ± SEM(n) of experiments run on n different preparations.

[Toxin] (nM)	β-Butx	*Naja naja atra* PLA_2
	Choline Uptake (% of control)	
0.001	103	104
0.01	90	-
0.1	71±2(5)	91±10(4)
1	58±4(4)	82± 9(4)
10	44	44;41

TABLE 27. ^{14}C-choline and ^{14}C-acetylcholine release from various bands on the Percoll gradient used to isolate mouse brain synaptosomes. Following a 30 min ^{14}C-choline (10 μM) uptake period (37°C), fractions were incubated for 5 min in buffer containing choline (10 μM) and neostigmine (4 μM) at 37°C with or without β-bungarotoxin (β-Butx; 0.5 μM) or high K^+ (40 mM) and radiolabeled choline and acetylcholine in the supernatant subsequently determined by radioimaging scanning of a TLC plate.

	Counts (mean ± SE; n=3 or duplicate values)					
	Control		High K^+		β-Butx	
Band	ACh	Ch	ACh	Ch	ACh	Ch
1	407±25	456±41	258;253	495;554	345;356	426;812
2	659±29	496±42	700;682	607;579	618;604	482;371
3	402±40	409±27	472;522	460;454	651;508	493;552
4	228;173	353;436	236;499	291;270	441;267	352;253

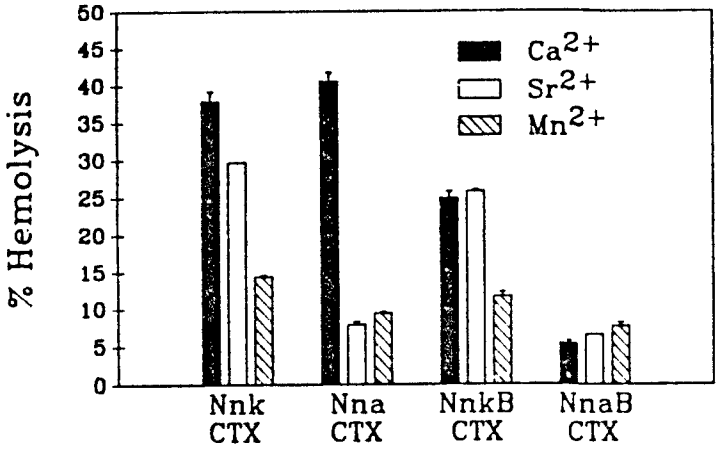
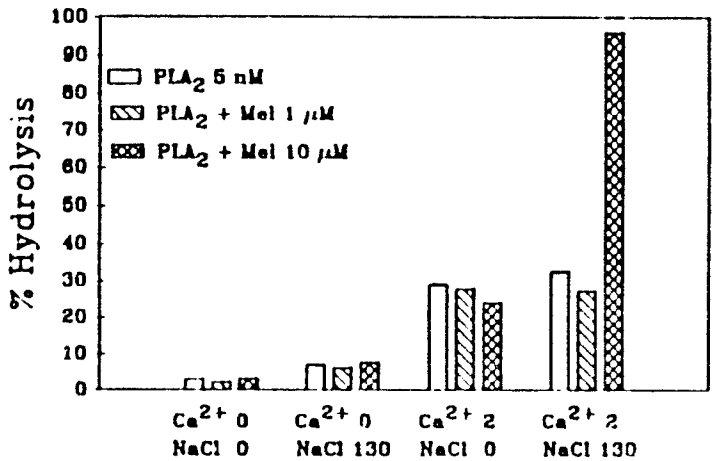


FIGURE 1
Hemolysis of aged (Red Cross cells past expiration date) erythrocytes by N. n. kaouthia and N. n. atra CTXs - effects o divalent cations and p-BPB-treatment. Re blood cells (0.5 ml packed cells in 1.0 ml incubation volume) were incubated in HEPES 20 mM, NaCl 130 mM at pH 7.4 and 37°C for 2 hr. The buffer contained a 2 mM concentration of Ca^{2+}, Sr^{2+} or Mn^{2+} and 30 µM toxin.

A.



B.

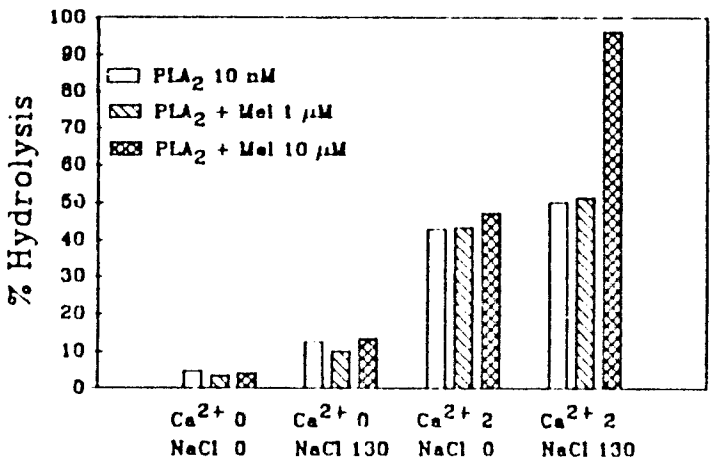
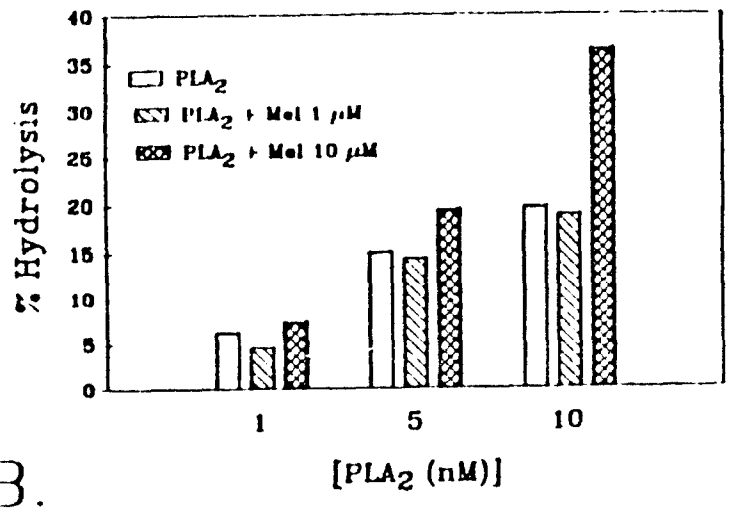


FIGURE 2
Effects of $CaCl_2$ and NaCl on the stimulation of bee venom PLA_2 activity o mixed micelle egg yolk PC substrates by p-BPB-treated melittin. Mixed micelles were of the same composition of PC as in Figure 1, with the addition of Triton X-100 (4 mM). Bee venom PLA_2 (Panel A = 5 nM; Panel B = 10 nM) was incubated for 3 min (37°C) in HEPES buffer with melittin in the following concentrations (in µM): open bars, 0; diagonal bars, 1 and; crosshatch bars, 10. The concentrations of $CaCl_2$ and NaCl are indicated (in mM) below each set of bars. Values are the average of duplicate determinations. The maximum difference between duplicates wa 4% hydrolysis.

A.



B.

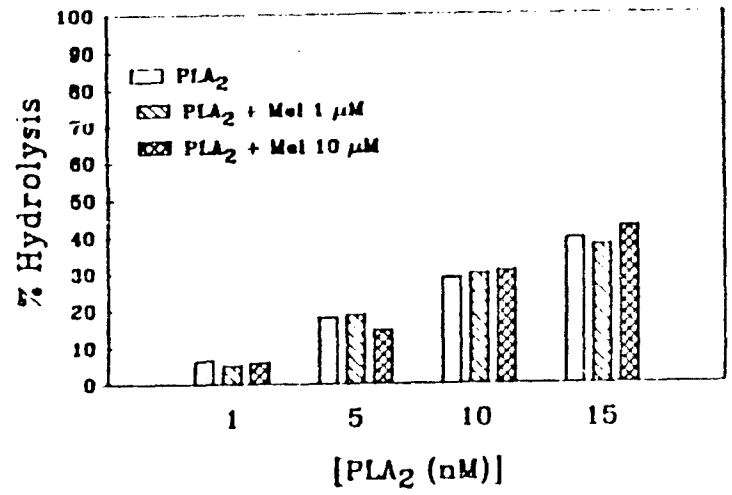


FIGURE 3

Influence of level of phospholipid hydrolysis on the stimulation of bee venom PLA_2 activity in mixed micelle egg yolk PC substrates by p-BPB-treated melittin. Mixed micelles were of the same composition as in Figure 6. Panel A: Various concentrations of PLA_2 (1-10 nM) were incubated for 2 hrs (37°C) in HEPES buffer with $CaCl_2$ (0 mM), NaCl (130 mM) and melittin in the following concentrations (in μM): open bars, 0; diagonal bars, 1 and; crosshatch bars, 10. Panel B: Various concentrations of PLA_2 (1-15 nM) were incubated for 30 min (37°C) in HEPES buffer with $CaCl_2$ (2 mM), NaCl (0 mM) and melittin in the same concentrations as in Panel A. Values are the average of duplicate determinations. The maximum difference between duplicates was 4% hydrolysis.

A.

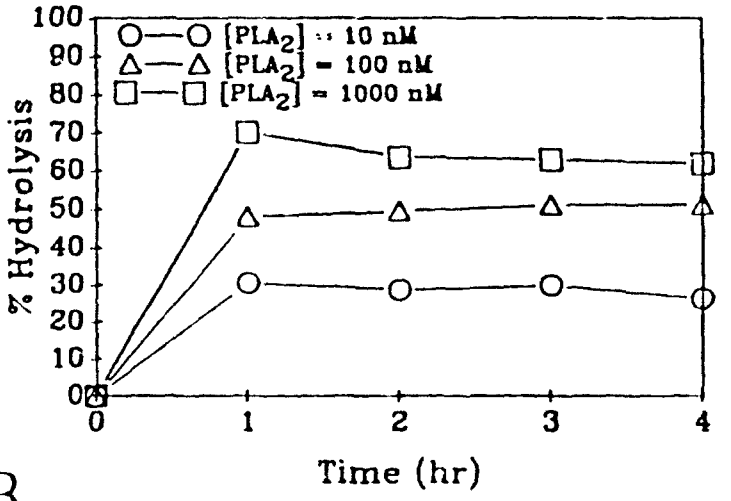


FIGURE 4
Hydrolysis of radiolabeled L-3-PC (1-stearoyl-2-[1-^{14}C]arachidonyl) (10 μM) in ULVs of egg yolk PC:Triton X-100 (1 mM:4 mM) by bee venom PLA_2. The concentrations of bee venom PLA_2 used were (in nM): circles, 10; triangles, 100; squares, 1000. Toxins were incubated for 1-4 hr at 37°C in HEPES buffer containing: (A) NaCl (130 mM) and $CaCl_2$ (2 mM), or; (B) no NaCl or $CaCl_2$. Values are the average of duplicate determinations, both of which are contained within the symbol.

B.

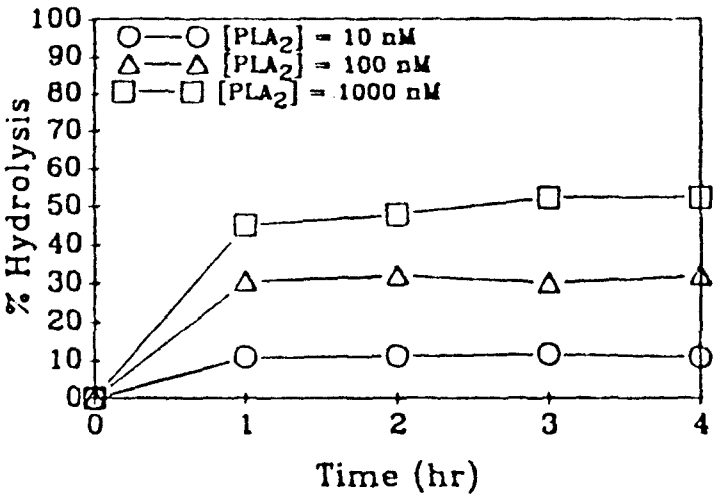
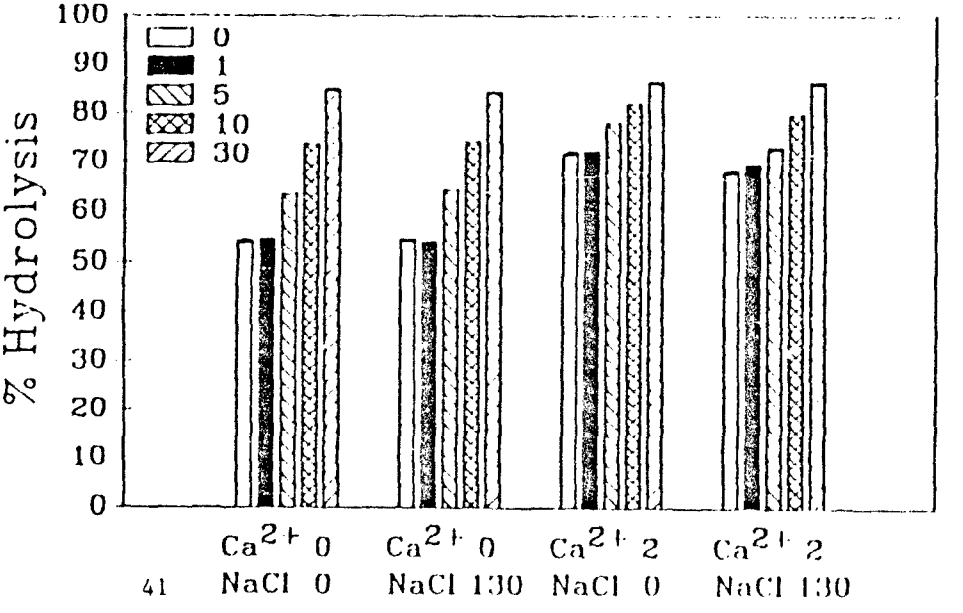


FIGURE 5
Effects of $CaCl_2$ and NaCl on the stimulation of bee venom PLA_2 activity on ULV egg yolk PC substrates by p-BPB-treated melittin. ULVs were of the same composition as in Figure 1. A fixed concentration of PLA_2 (1000 nM) was incubated for 2 hrs (37°C) in HEPES buffer with melittin in the following concentrations (in μM): open bars, 0; solid bars, 1; left diagonal bars, 5; crosshatch bars, 10 and; right diagonal bars, 30. The concentrations of $CaCl_2$ and NaCl are indicated (in mM) below each set of bars. Values are the average of duplicate determinations. The maximum difference between duplicates was 4% hydrolysis.



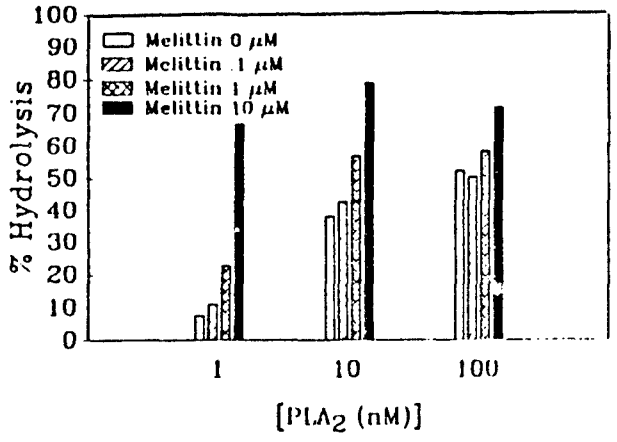


FIGURE 6
Stimulation of low concentrations of bee venom PLA_2 activity on ULV egg yolk PC substrates by p-BPB-treated melittin. ULVs were of the same composition as in Figure 1. Various concentrations of PLA_2 (1-100 nM) were incubated for 2 hrs (37°C) in HEPES buffer with $CaCl_2$ (2 mM), NaCl (130 mM) and melittin in the following concentrations (in μM): open bars, 0; diagonal bars, 0.1; crosshatch bars, 1 and; solid bars, 10. Values are the average of duplicate determinations. The maximum difference between duplicates was 3% hydrolysis.

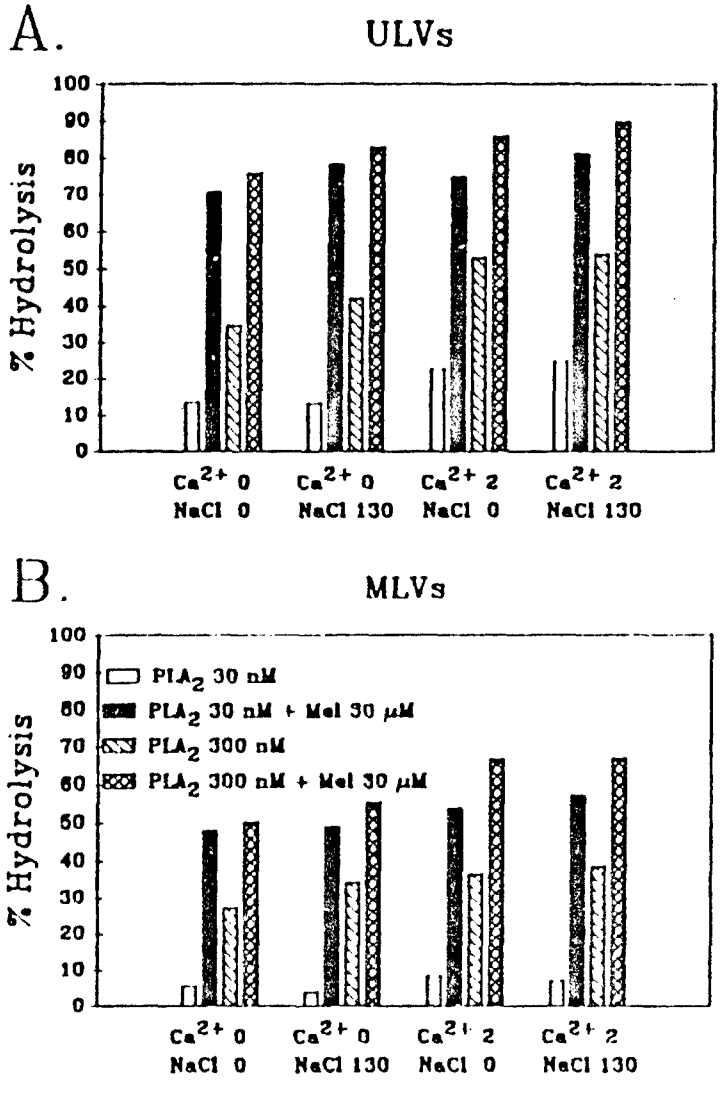


FIGURE 7
Effects of $CaCl_2$ and NaCl on the stimulation of submicromolar concentrations of bee venom PLA_2 in ULV and MLV egg yolk PC substrates by p-BPB-treated melittin. ULVs (Panel A) and MLVs (Panel B) were of the same composition as in Figure 1. Bee venom PLA_2 was incubated for 2 hrs (37°C) in HEPES buffer with melittin in the following concentrations (in μM): open bars, PLA_2 = 0.03 and melittin = 0; solid bars, PLA_2 = 0.03 and melittin = 30; diagonal bars, PLA_2 = 0.3 and melittin = 0; crosshatch bars, PLA_2 = 0.3 and melittin = 30. The concentrations of $CaCl_2$ and NaCl are indicated (in mM) below each set of bars. Values are the average of duplicate determinations. The maximum difference between duplicates was 4% hydrolysis.

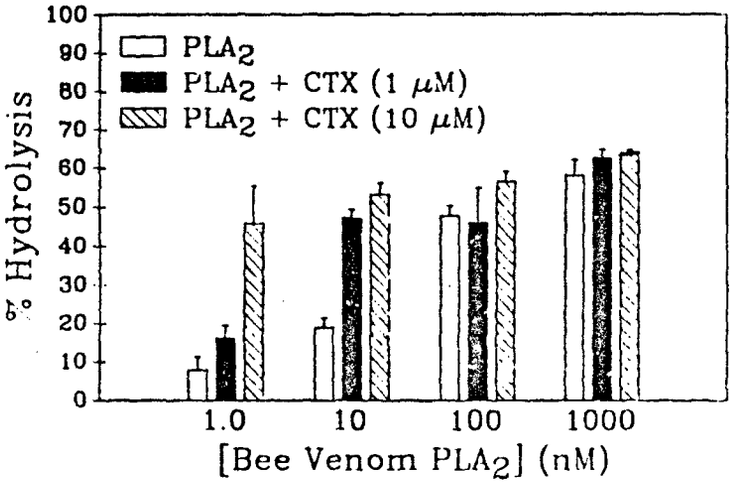
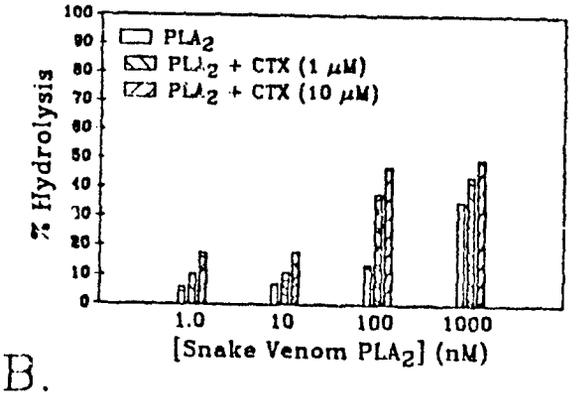


FIGURE 8
Concentration-dependence of p-BPB-treated CTX and bee venom PLA_2 interaction. ULVs, NaCl 130 mM, $CaCl_2$ 2 mM, 37°C, 2 hr. Mean ± SD (n=3).

A.



B.

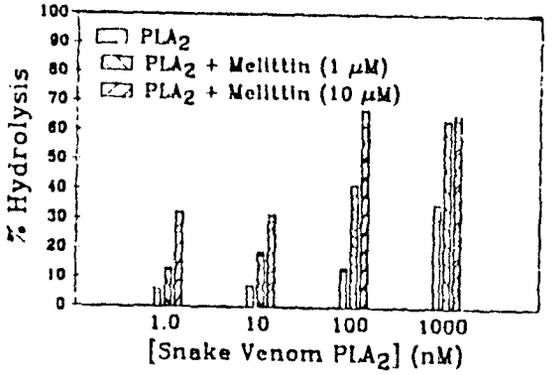


FIGURE 9
Concentration-dependence of p-BPB-treated CTX (A) or melittin (B) and snake venom PLA_2 interaction. ULVs, NaCl 130 mM, $CaCl_2$ 2 mM, 37°C, 2 hr.

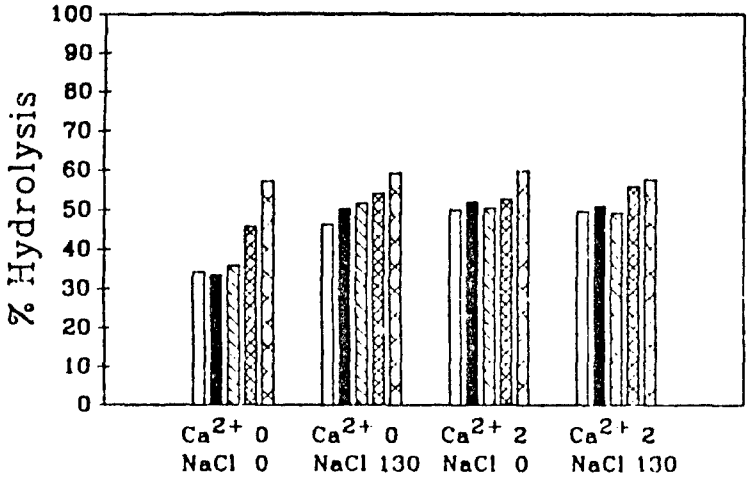


FIGURE 10

Effects of $CaCl_2$ and NaCl on the stimulation of 1 μM concentration of snake venom PLA_2 in ULV egg yolk PC substrates by p-BPB-treated CTX. ULVs were of the same composition as in Figure 1. Snake venom PLA_2 was incubated for 2 hrs (37°C) in HEPES buffer with CTX in the following concentrations (in μM): open bars, 0; solid bars, 1; diagonal bars, 5; narrow crosshatch bars, 10; wide crosshatch bars, 30. The concentrations of $CaCl_2$ and NaCl are indicated (in mM) below each set of bars. Values are the average of duplicate determinations. The maximum difference between duplicates was 4% hydrolysis.

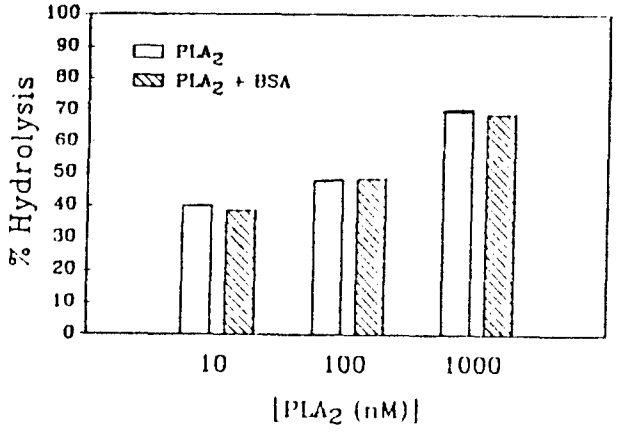


FIGURE 11

Effects of BSA on bee venom PLA_2 activity in ULV egg yolk PC substrates. ULVs were of the same composition as in Figure 1. Various concentrations of PLA_2 (10-1000 nM) were incubated for 2 hrs (37°C) in HEPES buffer with $CaCl_2$ (2 mM) and NaCl (130 mM). The open bars denote PLA_2 alone and the diagonal bars indicate the addition of BSA (0.5%). Values are the average of duplicate determinations. The maximum difference between duplicates was 2% hydrolysis.

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